REMARKS

I. General Remarks

Claim 1 has been amended to insert the "5-7 membered saturated heterocyclic ring" limitation of claim 2, and to remove sulfur as an atom that may interrupt the heterocyclic ring. Support for this amendment can be found, for example, in claim 2 as originally filed.

Claim 5 has been amended to correct a typographical error and to reword the claim in a manner requested by Examiner Nagubandi in an application (USSN 10/537,482) with similar claim language.

Claims 19, 21, 22 and 24 have been canceled without prejudice or disclaimer. These claims were directed to methods of treating a urological disorder or disease, an inflammatory disorder, urinary incontinence or overactive bladder, and asthma or COPD. Accordingly enablement issues regarding these indications have been rendered moot and are not addressed in this Response.

Claims 27 and 28 have been added, and recite two of the compounds that have been allowed in claim 4. As claim 4 has already been considered allowable, claims 27 and 28 are allowable and do not require additional searching by the Examiner.

Reconsideration of the remaining rejections is requested in view of the above amendments and the remarks below.

II. Examiner's Statements Regarding October 2, 2007 Interview

A. Misunderstanding regarding submission of *in-vivo* data

The Examiner stated that the allowability of the treatment of pain claims was contingent upon the submission of *in-vivo* data, and that the Examiner was under the impression that *in-vivo* data would be provided in the October 19, 2007 Response. Applicants do not recall representing that *in vivo* data would be submitted. Instead, Applicants recall stating that data acceptable to one of ordinary skill in the art is shown by the IC_{50} of capsaicin-induced Ca^{2+} influx in the human VR1-transected CHO cell line in light of the known correlation between TRPV1 antagonism and analgesia. Such correlation is shown, for example, in Exhibits S and T of the October 19, 2007 response which is discussed in detail below.

> The Examiner states:
>
> Mr. Madge had indicated that the compounds were tested in numerous prophetic animal assays in the instant specification, and the examiner was under the impression that such data would be made available to him. ***In the absence of such data, the examiner maintains the current rejections for the treatment of pain.***

(Office Action, page 2, paragraph 3, emphasis added).

Initially, Applicants note that Dr. Madge is a scientist at Xention Ltd. and the present application was initially filed on behalf of Bayer AG. Dr. Madge was not privy to details regarding development at Bayer AG and could not have definitively made such a representation.

Furthermore, Applicants inquire, in view of the bolded statement above, whether the Examiner has considered all of the evidence of enablement submitted in the previous Response, such as the correlation between analgesia and TRPV1 antagonism. Failure to consider all of the available evidence would not be in accordance with proper Examination procedure. MPEP § 2164.05 states that enablement shall be determined based on all the weight of all the evidence. MPEP § 2107.03 states that Office personnel should not impose on applicants the unnecessary burden of providing evidence from human clinical trials and that data generated using *in vitro* assays will be sufficient to establish therapeutic or pharmacological utility for a compound, composition or process if reasonably correlated to the particular therapeutic or pharmacological utility. Again, such correlation is provided by Exhibits S and T of the October 19, 2007 response along with additional data set forth in this Response.

The Office's policy that human or animal clinical data shall not be required is also sound public policy in this instance. As disclosed in the specification and claimed, these compounds have use in pain treatment. Thus, the requirement that animal testing be performed to ascertain analgesia would lead to pain and suffering. While clinical data is undoubtedly necessary for regulatory purposes, the MPEP makes clear that it is not necessary in the present case, especially given the established correlation of *in vitro* TRPV1 antagonism and analgesia.

While Applicants respectfully submit that the Examiner has not sufficiently addressed the *in vitro* data set forth in the specification along the correlation between TRPV1 antagonism and analgesia, any confusion resulting from the October 2, 2007 interview is regretted. Applicants appreciate the Examiner's remarks regarding the interview so that such

confusion can be clarified. The Examiner is urged to contact the undersigned if any confusion remains.

III. Objection to Claim 4

The Examiner states that claim 4 is objected to for depending from a rejected base claim, but would be allowable if "put in proper dependent format."

Applicants previously amended claim 4 in their October 19, 2007 response to be in independent form. Claim 4 no longer depends from a rejected claim and should have been allowed.

As noted above, claims 27 and 28 have been added. These claims depend from claim 4 and relate to N-[(7R)-7-hydroxy-5,6,7,8-tetrahydronaphthalen-1-yl]-N'-[3-pyrrolidin-1-yl-4-(trifluoromethyl)benzyl]urea and N-[(7R)-7-hydroxy-5,6,7,8-tetrahydronaphthalen-1-yl]-N'-[3-morpholin-4-yl-4-(trifluoromethyl)benzyl]urea. These two compounds are included in claim 4 and thus do not require additional searching by the Examiner.

IV. Rejections of the Claims Under 35 U.S.C. § 112, first paragraph -- Therapeutic Indications

A. Applicants Have Demonstrated the Correlation Between Analgesia and VR1 (TRPV 1) Antagonist

As noted above, Applicants have already demonstrated the correlation between pain relief and TRPV1 antagonism. For example, The Journal of Biological Chemistry, 280(14):13424-13432 (2005), Exhibit S of Applicants' October 19, 2007 response, explains in the first sentence:

> The vanilloid receptor TRPV1 is a polymodal nonselective cation channel of nociceptive sensory neurons involved in the perception of inflammatory pain

(Abstract, first sentence). The publication also notes studies on TRPV1 gene knock-out mice suggest that "TRPV1 is essential for the development of thermal hyperalgesia following inflammation or local injection of bradykinin and nerve growth factor." Based on the "essential" role that TRPV1 plays with the perception of pain, a person of ordinary skill would correlate TRPV1 antagonism with pain relief.

Exhibit T of the October 19, 2007 Response, entitled "Novel Non-Vanilloid VR1 Antagonists of High Analgesic Effects and Its Structural Requirement for VR1 Antagonistic Effect", Biorganic & Medicinal Chemistry Letters, 13:4389-4393 (2003) further supports the correlation between VR1 antagonism and analgesia. This reference corroborates the evidence discussed in Exhibit S that VR1 is involved with the perception of pain, noting:

> The idea that VR1 functions as an integrator of multiple pain-producing stimuli implies that VR1 antagonists or channel-blockers should have profound antinociceptive effects, especially in inflammatory pain models (page 4389, 2nd column)

Much like the compounds of the present application, the antagonistic effects of the subject compound, SC0030, was evaluated based on *in vitro* analysis of Ca^{2+} uptake inhibition (see p. 4390, 2nd column). SC0030 was found to be an inhibitor of Ca^{2+} uptake and thus a VR1 antagonist. SC0030 was also found to inhibit PBQ-induced writhing in mice, "which is considered to be indicative of an antinociceptive [analgesic] effect" (see p. 4390, 2nd column, last paragraph).

Based on the substantial evidence that TRPV1 receptors play on the perception of pain, as reported in Exhibits S and T, and the demonstration in Exhibit T that TRPV1 antagonism actually ameliorates pain, Applicants submit that one of ordinary skill in the art would find that TRPV1 antagonism is reasonably correlated with pain reduction.

B. Applicants Submit Herewith Additional Evidence Showing the Correlation between Analgesia and VR1 (TRPV1) Receptors

Attached as Exhibit A to this Response is the publication, "Vanilloid Receptor-1 is Essential for Inflammatory Thermal Hyperalgesia, " Nature, 405:183-187 (2000). As the title, suggests, this publication further corroborates the link between pain perception and VR1. The publication notes:

> findings and research linking capsaicin with nociceptive behaviours (that is responses to painful stimuli in animals) have led to VR1 being considered as important for pain sensation

(page 183, second column). The publication concludes that VR1 is required for inflammatory sensitization to noxious thermal stimuli (Id.).

Also attached as Exhibit B of this Response is a copy of the publication, entitled "The Cloned Capsaicin Receptor Integrates Multiple Pain-Producing Stimuli" Neuron, 21:531-543 (1998), which notes:

> VR1 can therefore be viewed as a molecular integrator of chemical and physical stimuli that elicit pain

(page 531, first column).

Exhibit C to this Response is the publication "The Vanilloid Receptor TRPV1: 10 Years from Channel Cloning to Antagonist Proof-of-Concept." This review provides a more recent summary of TRPV1 antagonist development which corroborates the earlier publications discussed above, explaining:

> TRPV1, perhaps the most important signal integrator in sensory nociceptors, is well established as an intriguing novel target for the treatment of pain. Extensive preclinical profiling of small molecule inhibitors of TRPV1 provides intriguing evidence that TRPV1 blockade can be a useful therapeutic approach for inflammatory, cancer and possibly neuropathic pain

(page 369, first column)

Third parties have developed other TRPV1 antagonists for the treatment of pain. Tables 1 and 2 provide TRPV1 antagonists and agonists that are currently under FDA review for use in the treatment of pain, further substantiating the well established link between the TRPV1 receptor and pain relief, and TRPV1 antagonists and pain relief.

A review of the patent literature further confirms the correlation between TRPV1 antagonism and such development. Third parties have obtained claims directed to methods of treating pain that include the step of administering a broad genus of TRPV1 antagonists.

For example, U.S. Patent No. 7,053,088, entitled "Vanilloid Receptor Ligands and Their Use in Treatments" is directed to treatment of vanilloid-receptor-mediated diseases with VR1 antagonists. The patent states that VR1 agonists and antagonists have use as analgesics for the treatment of pain of various genesis or etiology ('088 Patent, col. 2, lines 6-7). The specification states that vanilloid-receptor-mediated diseases include inflammatory or neuropathic pain, dental pain, general headache, migraine, cluster headache, tension headache, neuropathic pain and associated hyperalgesia and allodynia, diabetic neuropathy pain,

sympathetically maintained pain, trigeminal or herpetic neuralgia, causalgia, sympathetically maintained pain and deafferentiation syndromes such as brachial plexus avulsion.

Claims 6-8 of the '088 patent are specifically directed to treatment of acute, inflammatory and neuropathic pain, and involve administering the broad range of compounds recited in claims 1-3. It is noted that the '088 patent does confirm VR1 antagonistic behavior of various compounds via *in vitro* assays and *in vivo* testing data is not disclosed in the specification (see '088 patent, col. 124, line 34 - col. 127, line 4).

U.S. Patent No. 7,074,805, entitled "Fused Azabicyclic Compounds that Inhibit Vanilloid Receptor Subtype 1 (VR1) Receptor" is directed to VR1 antagonist compounds that treat pain (see abstract). Claim 65 of this patent is directed to methods of treating inflammatory thermal hyperalgesia, claim 68 is directed to treating pain and claim 69 is directed to inflammatory thermal hyperalgesia. This patent also contains *in vitro* data that, like the present application, confirms the VR1 antagonism of the claimed compounds (see '805 patent, col. 16, line 26 to col. 17, line 37).

U.S. Patent No. 6,939,891, entitled "Heterocyclic ureas, their preparation and their use as vanilloid receptor antagonists" also correlates VR1 antagonism and pain. The specification to the '891 patent states:

> The compounds of formula (I) and their pharmaceutically acceptable salts or pharmaceutically acceptable solvates have Vanilloid receptor antagonists (VR1) activity and are believed to be of potential use for the treatment or prophylaxis of certain diseases or disorders mediated or associated with the activity of vanilloid receptor, **including disorders such as pain, chronic pain, neuropathic pain, postoperative pain, rheumatoid arthritic pain, osteoarthritic pain, back pain, visceral pain, cancer pain, algesia, neuralgia, migraine, neuropathies, diabetic neuropathies, sciatica, HIV-related neuropathy, post-herpetic neuralgia, fibromyalgia, nerve injury . . .**

('891 patent, col. 4, lines 55-67, emphasis added). As with the '088 patent, the specification only provides *in vitro* data of the antagonist behavior of the claimed compounds ('891 patent col. 11, line 55 to col. 12, line 24). Notwithstanding, the '891 patent, like the '088 patent, teaches methods of treating pain ('891 patent, col. 5, lines 7-14). Similarly, claim 2 recites a method of treating pain. Claim 3 recites a method for the treatment of diseases or disorders mediated or

associated with activity of the vanilloid receptor and selected from, *inter alia*, pain, chronic pain, neuropathic pain, postoperative pain, rheumatoid arthritic pain, osteoarthritic pain, back pain, visceral pain, cancer pain, algesia, neuralgia, migraine, neuropathies, diabetic neuropathy, sciatica, HIV-related neuropathy, post-herpetic neuralgia, fibromyalgia, and nerve injury.

V. Rejections of the Claims Under 35 U.S.C. § 112, first paragraph -- Compound Synthesis

A. The Examiner has not provided sufficient basis to dismiss Methods B - H

In their October 19, 2007 response, Applicants went to great effort and expense to respond, point by point, to the Examiner's particularized allegations regarding Methods B-G of synthesizing the compounds of the present application. The Examiner does not acknowledge or respond to Applicants prior response to these allegations but merely asserts:

> The prophetic syntheses are just that and from a practical point of view are of little value.

Applicants request that the Examiner explain the basis for this statement in view of the October 19, 2007 response and the response below to the Examiner's assertions regarding Methods B-H. See, e.g., MPEP § 704.14(b) ("Information constituting answers to queries posed by the examiner or another Office employee must be considered, and the record must indicate that the answers were considered")

For example, the Examiner still has not indicated why Method H is regarded by him as inoperable. Applicants pointed this out in their October 19, 2007 response. The Examiner did not respond with any purported problems with Method H in the instant Office Action. Since the burden to establish lack of enablement is on the Examiner, Method H should be considered as enabled and is a method one of ordinary skill in the art could use to synthesize the instantly claimed compounds. See MPEP § 2164.04 ("it is incumbent upon the Patent Office. . . to explain why it doubts the truth or accuracy of any statement in a supporting disclosure . . . Otherwise, there would be no need for the applicant to go to the trouble and expense of supporting his presumptively accurate disclosure"); and *In re Fisher*, 427 F.2d 833, 839 (CCPA 1970) (claim enabled if specification discloses at least one method for a making and using claimed invention that bears reasonable correlation to the entire scope of the claim).

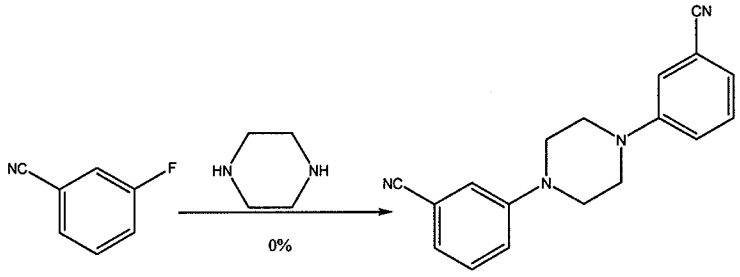
B. Method A is operable

While the Examiner appears to admit that the activating groups in the SNAr reaction need not be in the ortho or para position, the Examiner asserts that:

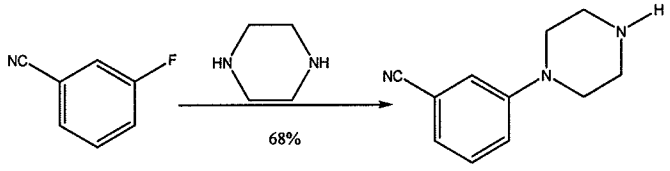
> reactions of the meta substituted compounds are unpredictable as stated by the applicant remarks at 30: 'Exhibit M notes that the following proposed reaction had a zero percent yield (referring to the reaction of a meta substituted nitrile)

(Office Action, page 4).

The reaction that had a zero percent yield was:



Instead, Exhibit M states that the following reaction takes place:



:

However, if an equimolar mixture of this reaction product and 3-fluorobenzonitrile is allowed to react with 3 equivalents of K_2CO_3, the same product as set forth above for the 0% yield reaction may be formed:

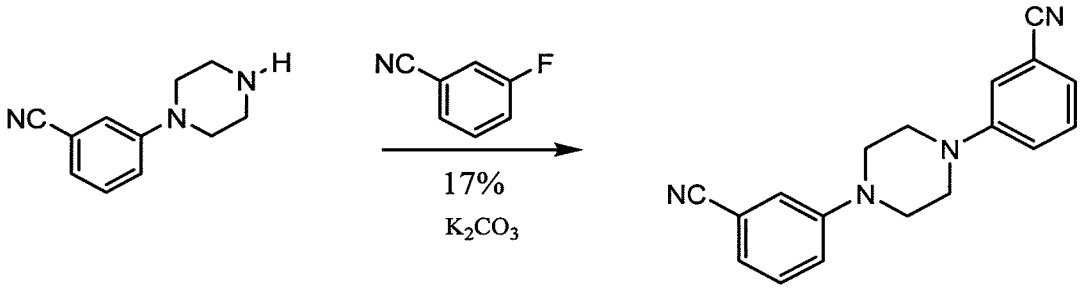


Exhibit M does not show that "reactions of the meta substituted compounds are unpredictable". Rather, it demonstrates that, contrary to the Examiner's original position when asserting that Example 1-25 is not enabled, piperazines will not necessarily become derivatized at both nitrogens when reacting with a meta substituted benzonitrile. Whether or not this is "predictable" is not discussed in Exhibit M and is not relevant for this question of enablement; Applicants have shown that the compound corresponding to 3SM that is used to form the compound of Example 1-25 can be prepared. Exhibit M certainly does not give basis for the generalization that the Examiner has made in stating that the reactions of meta substituted compounds are unpredictable and, in any case, does not suggest that the desired compounds (*i.e.* the 3SM compounds) could not be arrived at by reasonable trial and error.

> The Examiner also asserts:
>
> While no doubt the entire scope of the claims is not enabled, the main point of contention after the telephone interview was the nature of the substituent amino heterocycle (NR_2R_3).

Applicants address the NR_2R_3 substituent below, but respectfully disagree with the Examiner's implication that other, unstated issues remain with respect to enablement. As noted above, the Applicants have previously responded to each of the particularized assertions of the Examiner. The Examiner has either agreed with the Applicants (See Office Action, top of page 6) or has not met his burden of *directly* addressing these replies. Accordingly, Applicants submit that, based on the record, the entire scope of the claims is enabled.

The Examiner cites a treatise noting that "non-chemists would probably be horrified if they were to learn how many attempted syntheses fail" (*Side Reactions in Organic*

Synthesis, 2005, Wiley: VCH). The reference concludes that "[s]mall structural variations of polyfunctional substrates might, therefore, bring about an unforeseeable change in reactivity".

Applicants respectfully submit that the passage cited by the Examiner has no relevance to the instant application. First, the passage does not establish whom the person of ordinary skill in the art is. In the April 19, 2007 Office Action, the Examiner stated that:

> The level of ordinary skill in the art is *extremely* high. Typical researchers have a Ph.D. in organic chemistry, post-doctoral training and *many* years of experience in diverse fields.

(Office Action dated April 19, 2007, page 26, line 7). Furthermore the text relied upon by the Examiner is not in the context of this *extremely* skilled artisan with *many* years of experience having the benefit of the present application, which discloses 8 synthesis techniques. Indeed, the passage is directed to an irrelevant person, i.e., a non-chemist. Any chemist would know that not every attempted synthesis will succeed, just as well as he or she knows that success requires routine trial and error.

The Examiner also relies upon a case (*Automotive Technologies Intern. v. BMW of North America, Inc.*, 501 F.3d 1274 (Fed. Cir. 2007)) regarding mechanical and electronic side impact sensors, apparently to again suggest that the specification does not enable the full scope of the claims. As noted above, Applicants respectfully disagree with this assertion, particularly given that the Examiner has not acknowledged the responses to the Examiner previous assertions regarding the synthesis-based enablement concerns of the present application.

C. To advance prosecution, the definition of -NR_2R_3 has been amended

As noted above, claim 1 has been amended to insert the "5-7 membered saturated heterocyclic ring" limitation of claim 2, and to remove sulfur as an atom that may interrupt the heterocyclic ring. This amendment has been solely to advance prosecution, as Applicants respectfully disagree with the position taken by the Examiner that the definition of NR_2R_3 is too broad.

The Examiner has proposed in Table 1 on page 9 of the Office Action structures of unknown and/or theoretical molecules that would have previously been encompassed by the -NR_2R_3 substitutent. These speculative structures are moot in view of amended claim 1.

Applicants respectfully submit that specification enables the full range of the presently claimed -NR_2R_3 substituents. The examples disclose:

5-membered heterocyclic rings (see, e.g., example nos. 1-1, 1-7, 1-8, 1-9, 1-13, 1-14, 1-16 and 1-17);

6-membered heterocyclic rings (see, e.g., examples nos. 1-2, 1-3, 1-4, 1-5, 1-6, 1-15, 1-18, 1-19 , 1-20, 1-21, 1-22, 1-23, 1-24, 1-25, 1-26 and 1-28);

7-membered heterocyclic rings (see e.g., example nos. 1-10, and 1-11);

uninterrupted heterocyclic rings (see e.g., example nos. 1-1, 1-2, 1-5, 1-7, 1-8, 1-9, 1-10, 1-11, 1-13, 1-14, 1-15, 1-16, 1-17, 1-19, 1-20, 1-21, 1-22, 1-23, and 1-24);

O-interrupted heterocyclic rings (see e.g., example nos. 1-3, 1-4, 1-6, 1-26, and 1-28);

N-interrupted heterocyclic rings (see e.g., example nos. 1-18, and 1-25);

substituted heterocyclic rings (see e.g., example nos. 1-13, 1-18, 1-20, 1-21, 1-22, 1-23, and 1-24); and

exemplary compounds in which R_2 is an alkylamino group and R_3 is hydrogen (see, e.g., example no. 1-12), and in which R_2 is and alkyl group and R_3 is an alkyl group substituted with a hydroxy group (see, e.g., example no. 1-27).

The applications disclosure is sufficient, particularly in view of governing case law such as *In re Angstadt,* 537 F.2d 498 (CCPA 1976), which explains:

> The question, then, is whether in an unpredictable art, section 112 requires disclosure of a test with every species covered by a claim. To require such a complete disclosure would apparently necessitate a patent application or applications with "thousands" of examples or the disclosure of "thousands" of catalysts along with information as to whether each exhibits catalytic behavior resulting in the production of hydroperoxides. More importantly, such a requirement would force an inventor seeking adequate patent protection to carry out a prohibitive number of actual experiments. This would tend to discourage inventors from filing patent applications in an unpredictable area since the patent claims would have to be limited to those embodiments which are expressly disclosed.

* * *

> We hold that the evidence as a whole, including the inoperative as well as the operative examples, negates the PTO position that persons of ordinary skill in this art, given its unpredictability, must engage in undue experimentation to determine which complexes work. The key word is "undue," not "experimentation." See Fields v. Conover, supra.

Id. at 502-504 Applicants respectfully submit that the claimed $-NR_2R_3$ group, which may invoke "experimentation," is not undue. Accordingly, this claim is enabled in view of the application's disclosure.

VI. Remarks Regarding Provisional Double-Patenting Rejections

Claims 1-3, 5, 7, and 19-26 are provisionally rejected on the ground of nonstatutory double patenting over claims 1-4 and 8-20 of copending application no. 10/513,848. Submitted herewith, solely to advance prosecution, is a terminal disclaimer over the '848 application.

Claims 1-3, 5, 7, 19-26 stand provisionally rejected on the ground of nonstatutory double patenting over claims 1-4 and 8-20 of copending application no. 10/575,027. Applicants submit that this is the last remaining issue of patentability and that the '027 application was filed after the present application. Applicants request that this rejection be withdrawn pursuant to MPEP § 804.I.B.1 which provides:

> If a "provisional" nonstatutory obviousness-type double patenting (ODP) rejection is the only rejection remaining in the earlier filed of the two pending applications, while the later-filed application is rejectable on other grounds, the examiner should withdraw that rejection and permit the earlier-filed application to issue as a patent without a terminal disclaimer.

Accordingly, Applicants request that this rejection be withdrawn.

VII. No Waiver

All of Applicants' arguments and amendments are without prejudice or disclaimer. Additionally, Applicants have merely discussed example distinctions from the position taken by the Examiner. Other distinctions exist, and Applicants reserve the right to discuss these additional distinctions in a later Response or on Appeal, if appropriate. By not responding to additional statements made by the Examiner, Applicants do not acquiesce to the Examiner's additional statements. The example distinctions discussed by Applicants are sufficient to overcome the Examiner's rejections.

SUMMARY

In light of the above remarks, Applicants respectfully request reconsideration and withdrawal of the outstanding rejections. Applicants further submit that the application is now in condition for allowance, and earnestly solicits timely notice of the same. Should the Examiner have any questions, comments or suggestions in furtherance of the prosecution of this application, the Examiner is invited to contact the attorney of record.

Applicants believe that there are no fees due in association with this filing of this Response, apart from the fee for filing a terminal disclaimer. However, should the Commissioner deem that any additional fees are due, including any fees for extensions of time, the Commissioner is authorized to debit Baker Botts L.L.P. Deposit Account No. 02-0383, Order Number 078503.0104, for any underpayment of fees that may be due in association with this filing.

Respectfully submitted,

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EXHIBIT S OF

10/19/2007

RESPONSE

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Regulation of Ca^{2+}-dependent Desensitization in the Vanilloid Receptor TRPV1 by Calcineurin and cAMP-dependent Protein Kinase*

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The vanilloid receptor TRPV1 is a polymodal nonselective cation channel of nociceptive sensory neurons involved in the perception of inflammatory pain. TRPV1 exhibits desensitization in a Ca^{2+}-dependent manner upon repeated activation by capsaicin or protons. The cAMP-dependent protein kinase (PKA) decreases desensitization of TRPV1 by directly phosphorylating the channel presumably at sites Ser^{116} and Thr^{370}. In the present study we investigated the influence of protein phosphatase 2B (calcineurin) on Ca^{2+}-dependent desensitization of capsaicin- and proton-activated currents. By using site-directed mutagenesis, we generated point mutations at PKA and protein kinase C consensus sites and studied wild type (WT) and mutant channels transiently expressed in HEK293t or HeLa cells under whole cell voltage clamp. We found that intracellular application of the cyclosporin A·cyclophilin A complex (CsA·CyP), a specific inhibitor of calcineurin, significantly decreased desensitization of capsaicin- or proton-activated TRPV1-WT currents. This effect was similar to that obtained by extracellular application of forskolin (FSK), an indirect activator of PKA. Simultaneous applications of CsA·CyP and FSK in varying concentrations suggested that these substances acted independently from each other. In mutation T370A, application of CsA·CyP did not reduce desensitization of capsaicin-activated currents as compared with WT and to mutant channels S116A and T144A. In a double mutation at candidate protein kinase C phosphorylation sites, application of CsA·CyP or FSK decreased desensitization of capsaicin-activated currents similar to WT channels. We conclude that Ca^{2+}-dependent desensitization of TRPV1 might be in part regulated through channel dephosphorylation by calcineurin and channel phosphorylation by PKA possibly involving Thr^{370} as a key amino acid residue.

The capsaicin receptor TRPV1, a nonselective cation channel expressed predominantly in nociceptive sensory neurons, transduces and integrates various stimuli such as noxious heat (>42 °C), capsaicin, protons, (1, 2), the endogenous cannabinoid anandamide (3), lipoxygenase products, and other lipids related to arachidonic acid (4) and ethanol (5). Studies on TRPV1 gene knock-out mice suggest that TRPV1 is essential for the development of thermal hyperalgesia following inflammation or local injection of bradykinin and nerve growth factor (6–8).

Activation of TRPV1 leads to Ca^{2+} influx into nociceptive sensory neurons, resulting in membrane depolarization and release of proinflammatory neuropeptides from primary afferent nerve terminals (9). Prolonged or repeated activation of TRPV1 results in desensitization and insensitivity of the receptor to subsequent stimuli (10, 11). The physiological role and importance of TRPV1 desensitization is unknown but speculated to be a process of adaptation and regulation of the peripheral nervous system for the perception of pain. Comparable with other ion channels, desensitization of TRPV1 is at least in part a Ca^{2+}-dependent process (10, 11). There is growing evidence for the involvement of Ca^{2+}-dependent phosphorylation and dephosphorylation processes to regulate desensitization and excitability of TRPV1. Previous studies in rat dorsal root ganglion neurons have demonstrated that desensitization is reduced in the presence of inhibitors of the Ca^{2+}- and calmodulin-dependent protein phosphatase 2B (calcineurin) (12). Conversely, phosphorylation of TRPV1 by Ca^{2+}-calmodulin-dependent kinase II (CaMKII)[1] seems to be a prerequisite for activation of TRPV1 by capsaicin (13).

Another candidate involved in the mechanisms of Ca^{2+} negative feedback and Ca^{2+}-dependent inactivation in many ion channels is the Ca^{2+} sensor calmodulin (CaM) itself. There is growing evidence that multiple regions of TRPV1 indeed may bind CaM (14, 15).

TRPV1 is also a target for cAMP-dependent protein kinase (PKA)- and protein kinase C (PKC)-dependent phosphorylation. Phosphorylation by PKA sensitizes the channel to heat (16) and capsaicin (17) and reduces Ca^{2+}-dependent desensitization of capsaicin- and proton-activated currents (18, 19). Amino acids residues Ser^{116} and Thr^{370} are the major substrates for PKA-dependent phosphorylation, although other putative PKA phosphorylation sites might be involved as well. Phosphorylation by PKC sensitizes the channel to capsaicin, protons, and heat (20–22). Here, residues Ser^{502} and Ser^{800} are the major substrates for PKC-dependent phosphorylation.

In the present study we investigated the influence of cal-

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[1] The abbreviations used are: CaMKII, Ca^{2+}-calmodulin-dependent kinase II; CaM, calmodulin; PKA, cAMP-dependent protein kinase; PKC, protein kinase C; WT, wild type; CsA, cyclosporin A; CyP, cyclophilin A; CsA·CyP, cyclosporin A·cyclophilin A complex; FSK, forskolin; HEK, human embryonic kidney; PMA, phorbol 12-myristate 13-acetate; OA, okadaic acid; W-7, *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide hydrochloride.

cineurin on Ca^{2+}-dependent desensitization of capsaicin- and proton-activated currents and examined the interactions of calcineurin and PKA and PKC phosphorylation pathways. We found that Ca^{2+}-dependent desensitization of TRPV1 might be in part regulated through channel dephosphorylation by calcineurin and channel phosphorylation by PKA possibly involving Thr^{370} as a key amino acid residue.

EXPERIMENTAL PROCEDURES

Site-directed Mutagenesis and Transient Transfection—Mutagenesis of rat TRPV1-cDNA was performed with rTRPV1-pcDNA3 by means of the transformer site-directed mutagenesis kit (BD Biosciences Clontech, Palo Alto, CA) as described previously (19). Human embryonic kidney (HEK) 293t cells or cells of a human adenocarcinoma-derived cell line (HeLa cells) were transfected with wild type or mutant plasmid (0.75 or 10 μg, respectively) along with reporter plasmid (CD8-pih3m, 1 μg) by the calcium phosphate precipitation method. After incubation for 12–15 h, the cells were replated in 35-mm culture dishes. Transfected cells were used for experiments within 2–3 days. Transfection-positive cells were identified by immunobeads (anti-CD-8 Dynabeads; Dynal Biotech, Oslo, Norway). Transfection efficiency was ~50–70% on average for TRPV1-WT and mutant channels.

Chemicals and Solutions—Capsaicin (8-methyl-*N*-vanillyl-6-nonenamide) and cyclosporin A (CsA; both Sigma-Aldrich) were dissolved in absolute ethanol to give stock solutions of 10 mM. Forskolin (FSK; Calbiochem-Novabiochem GmbH, Bad Soden, Germany), phorbol 12-myristate 13-acetate (PMA; Calbiochem-Novabiochem GmbH), and okadaic acid (OA; Alomone Labs, Ltd., Jerusalem, Israel) were dissolved in dimethyl sulfoxide to give stock solutions of 10, 1, and 1 mM, respectively. Human brain CaM and *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide hydrochloride (W-7; both Calbiochem-Novabiochem GmbH) were dissolved in double distilled water to give stock solutions of 100 mM. Cyclophilin A (CyP; Sigma-Aldrich) was dissolved in Tris-Cl, pH 7.4, containing HEPES, 1,4-dithio-DL-threitol, phenylmethanesulfonyl fluoride, and sodium azide to give a stock solution of 20 μM. All of the stock solutions were stored at −20 °C. All of the control and test solutions were applied with a polytetrafluorethylen glass multiple-barrel perfusion system. Standard bath solutions contained 70 mM NaCl, 70 mM choline Cl, 5 mM KCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM HEPES, and 10 mM glucose (adjusted to pH 7.4 with tetramethylammonium hydroxide). The NaCl/Choline Cl composition was used to reduce the amplitude of the WT currents. Choline Cl did not have any influence on WT or mutant channels. Pipette solution contained 140 mM KCl, 2 mM $MgCl_2$, 5 mM EGTA, and 10 mM HEPES (adjusted to pH 7.4 with KOH).

Electrophysiological Technique and Data Analysis—Currents were recorded at room temperature with the whole cell configuration of the patch-clamp method. Holding potential was −60 mV. Patch pipettes were pulled from boroslilicate glass tubes (TW150F-3; World Precision Instruments, Sarasota, FL) and heat-polished at the tip to give a resistance of 0.8- 1.2 MΩ. The currents were recorded with an Axopatch 200B patch-clamp amplifier (Axon Instruments, Union City, CA), filtered at 1 kHz, and sampled at 2 kHz. pCLAMP 8.0.1 software (Axon Instruments) was used for acquisition and analysis of currents. Origin 6.1 software (OriginLab Corporation, Northampton, MA) was used to perform least squares fitting and to create figures. The data are presented as the means ± S.E. or fitted value ± S.E. of the fit. An unpaired Student's *t* test (SigmaStat; SSPS Science, Chicago, IL) was used to evaluate the significance of changes in mean values. *p* values <0.05 were considered statistically significant.

RESULTS

Inhibition of Calcineurin Decreases Ca^{2+}-dependent Desensitization of Capsaicin-activated TRPV1-WT Currents—TRPV1 channel exhibits desensitization in a Ca^{2+}-dependent manner (10, 11). It has been suggested that a rise in cytosolic Ca^{2+} level caused by TRPV1 activation results in the activation of Ca^{2+}/calmodulin-dependent protein phosphatases that mediate channel desensitization (12). To test this hypothesis for TRPV1-WT transiently expressed in HEK293t cells, we first studied the effect of various protein phosphatase inhibitors on Ca^{2+}-dependent desensitization of capsaicin-activated TRPV1-WT currents, specifically on the decreasing current response to successive stimulation (tachyphylaxis). We applied

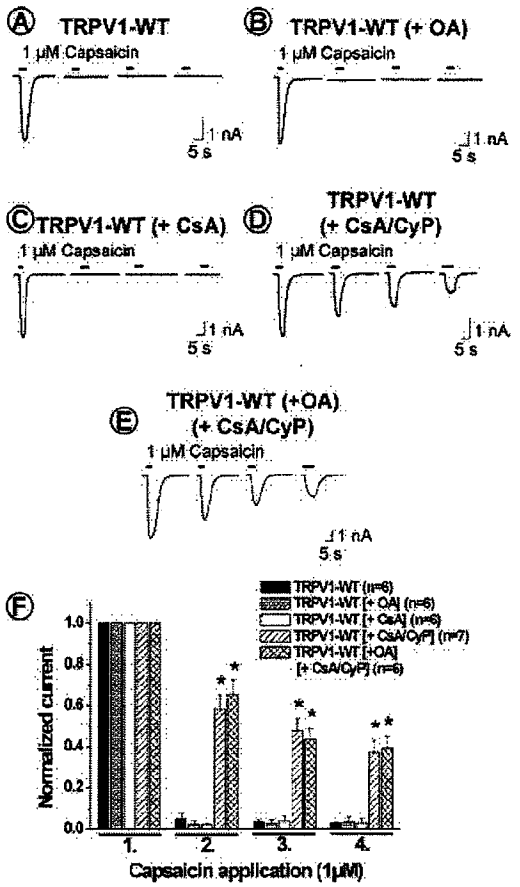

FIG. 1. **Effect of OA, a specific inhibitor of protein phosphatases 1 and 2A, CsA, and CsA·CyP, a specific inhibitor of protein phosphatase 2B (calcineurin), on Ca^{2+}-dependent desensitization of capsaicin-activated TRPV1 currents.** Shown are whole cell current responses of TRPV1 channels to repeated brief (~5 s long) applications of 1 μM capsaicin in Ca^{2+}-containing (2 mM) bath solution without (*A*) or with 1 μM OA (*B*), 100 μM CsA (*C*), or 14 nM CsA + 17 nM CyP in the pipette solution (*D*), or 14 nM CsA + 17 nM CyP in the pipette solution along with 1 μM OA applied extracellularly (*E*). After a whole cell voltage clamp was established, the cells were dialyzed for 10 min before the first capsaicin application. The intervals between capsaicin applications were 2 min. *F*, mean amplitudes of currents ± S.E. measured in experiments as described for *A–E*. The amplitudes were normalized to the current amplitude obtained with first capsaicin application. * indicates a statistically significant difference in the mean amplitude compared with that obtained under control conditions.

a series of brief (~5 s long) pulses of 1 μM capsaicin at 2-min intervals in Ca^{2+}-containing solution (2 mM) and measured the current response. Under control conditions, TRPV1-WT showed strong tachyphylaxis (Fig. 1*A*). Most of the tachyphylaxis occurred between the first and second application, as described previously (19). The mean current amplitudes at the second and fourth capsaicin application were 5.1 ± 2.7 and 3.1 ± 0.4% of that of the first application, respectively (Fig. 1*F*). Pretreatment of cells for 10 min with 1 μM OA in the pipette solution, an inhibitor of protein phosphatase 1 and 2A, did not lead to any change in Ca^{2+}-dependent tachyphylaxis of capsaicin-activated currents (Fig. 1*B*). Here, current amplitudes at the second and fourth capsaicin application were 2.5 ± 1.6 and 3.7 ± 2.4% of that of the first application, respectively (Fig. 1*F*). Similarly, OA was without any effect on Ca^{2+}-dependent tachyphylaxis when used in both lower or higher concentrations (0.01–100 μM, data not shown). In contrast, tachyphylaxis was significantly decreased when cells were pretreated for 10 min

TABLE I

Mean amplitudes of currents (means ± S.E.) evoked by the first capsaicin/proton application in n experiments under various experimental conditions

$p < 0.05$ indicates a statistically significant difference in the mean amplitude compared with that obtained under control conditions.

Channel	Activator	Experimental condition	Response	*n*	*p*
			nA		
TRPV1-WT	Capsaicin		6.5 ± 1.6	6	
TRPV1-WT	Capsaicin	100 μM OA in pipette	9.3 ± 2.6	6	
TRPV1-WT	Capsaicin	100 μM CsA in pipette	6.8 ± 1.2	6	
TRPV1-WT	Capsaicin	14 nM CsA, 17 nM CyP in pipette	4.8 ± 1.2	7	
TRPV1-WT	Capsaicin	100 μM OA plus 14 nM CsA, 17 nM CyP in pipette	4.3 ± 1.2	6	
TRPV1-WT	Capsaicin (30 s)		7.8 ± 1.8	6	
TRPV1-WT	Capsaicin (30 s)	14 nM CsA, 17 nM CyP in pipette	5.0 ± 1.1	7	
TRPV1-WT	Capsaicin	100 μM W-7 in pipette	7.8 ± 1.7	8	
TRPV1-WT	Capsaicin	100 μM CaM in pipette	8.6 ± 2.0	6	
TRPV1-WT	Capsaicin	10 μM FSK in extracellular buffer	5.2 ± 0.9	9	
TRPV1-S116A	Capsaicin		8.9 ± 2.3	7	
TRPV1-S116A	Capsaicin	14 nM CsA, 17 nM CyP in pipette	6.1 ± 1.2	7	
TRPV1-T370A	Capsaicin		2.3 ± 0.6	6	<0.05
TRPV1-T370A	Capsaicin	14 nM CsA, 17 nM CyP in pipette	3.2 ± 0.8	6	
TRPV1-T144A	Capsaicin		2.8 ± 0.6	6	
TRPV1-T144A	Capsaicin	14 nM CsA, 17 nM CyP in pipette	3.9 ± 1.0	6	
TRPV1-WT	Capsaicin	0.1 μM PMA in extracellular buffer	5.3 ± 2.3	6	
TRPV1-S502A/S800A	Capsaicin		6.2 ± 2.3	6	
TRPV1-S502A/S800A	Capsaicin	14 nM CsA, 17 nM CyP in pipette	4.6 ± 1.2	8	
TRPV1-WT	Proton		3.1 ± 0.6	6	
TRPV1-WT	Proton	14 nM CsA, 17 nM CyP in pipette	4.2 ± 0.7	6	
TRPV1-WT	Proton (30s)		6.4 ± 1.5	6	
TRPV1-WT	Proton (30s)	14 nM CsA, 17 nM CyP in pipette	4.0 ± 0.8	6	

with the immunosuppressive drug cyclosporin A (CsA; 14 nM) together with the "immunophilin" cyclophilin A (CyP; 17 nM) in the pipette solution (Fig. 1*D*). CsA and CyP are known to form a drug/immunophilin complex, which associates with and thus inhibits protein phosphatase 2B (calcineurin) (23). In the presence of CsA/Cyp, current amplitudes at the second and fourth capsaicin application were 58.4 ± 6.9 and 37.2 ± 6.2% of that at the first application, respectively (Fig. 1*F*). This is in good agreement with previously reported results obtained in rat dorsal root ganglion neurons using similar concentration of CsA and CyP in the pipette solution (12). Higher concentrations of CsA (up to 1 μM) along with CyP (up to 1 μM) did not lead to any further decrease in tachyphylaxis compared with that observed with 14 nM CsA plus 17 nM CyP (data not shown). Pretreatment of cells with CsA alone up to concentrations of 100 μM in the pipette solution did not lead to any change in Ca^{2+}-dependent tachyphylaxis of capsaicin-activated currents compared with control conditions, indicating that indeed CsA complexed to CyP is the active form that specifically targets calcineurin (Fig. 1, *C* and *F*). Pretreatment of cells for 10 min with 14 nM CsA and 17 nM CyP along with 1 μM OA in the pipette solution did not lead to any further decrease in tachyphylaxis compared with that observed with CsA·CyP (Fig. 1, *E* and *F*). Pretreatment of cells with OA, CsA, or CsA·CyP in the pipette solutions did not have any significant effect on capsaicin-activated peak currents of TRPV1-WT (Table I).

We also investigated responses of TRPV1-WT channels to prolonged applications (30-s) of 1 μM capsaicin in Ca^{2+}-containing (2 mM) bath solution. TRPV1-WT currents had peak amplitudes in the range of 2.6–14.4 nA with a mean of 7.1 ± 1.8 nA. Currents reached their peak at 1.6 ± 0.5 s after beginning of activation. The currents then began to decrease rapidly during continuous capsaicin application and reached values of 0.41 ± 0.18, 0.34 ± 0.06, and 0.21 ± 0.03 nA after 10, 20, and 30 s, respectively (Fig. 2*A*). This type of desensitization has been termed acute desensitization, consistent with previous reports (10, 11, 19).

Pretreatment of cells for 10 min with 14 nM CsA plus 17 nM CyP in the pipette solution also led to a significant decrease in acute desensitization of capsaicin-activated TRPV1-WT currents. The currents had peak amplitudes in the range of 2.4-8.9 nA with a mean of 5.0 ± 1.1 nA, which was not significantly different from control conditions (Table I). The currents reached their peak at 1.9 ± 0.7 s after beginning of activation. Then the currents began to decrease during continuous capsaicin application and reached values of 2.18 ± 0.62, 0.64 ± 0.08, and 0.59 ± 0.05 nA after 10, 20, and 30 s, respectively (Fig. 2*B*). To describe and compare acute desensitization quantitatively for control conditions and in the presence of CsA·CyP, we measured the areas under the current curves over a time of 30 s and normalized them to an idealized (rectangular), nondesensitizing current of a respective size. The data are given in Fig. 2*C*.

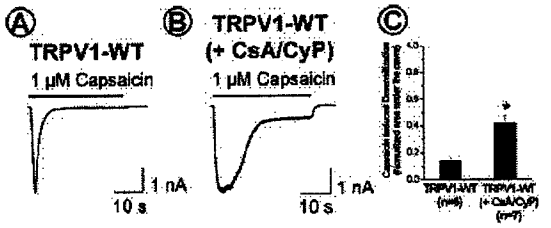

FIG. 2. **Effect of CsA complexed to CyP on acute desensitization of capsaicin-activated TRPV1 currents.** Shown are whole cell current responses to 30-s-long applications of 1 μM capsaicin in Ca^{2+}-containing (2 mM) bath solution without (*A*) or with 14 nM CsA +17 nM CyP in the pipette solution (*B*). After a whole cell voltage clamp was established, the cells were dialyzed for 10 min before the first capsaicin application. *C*, the areas under the current curves were measured and normalized to an idealized, nondesensitizing current of respective size. The *bars* represent mean values ± S.E. * indicates a statistically significant difference compared with control.

Calmodulin Is Not Required for the Decrease in Ca^{2+}-dependent Desensitization by Inhibition of Calcineurin—Calcineurin exerts its phosphatase activity in a Ca^{2+}- and calmodulin-dependent manner. CaM itself is a dominant Ca^{2+} sensor for Ca^{2+}-dependent inactivation in many ion channels (24–26). There is accumulating evidence that multiple regions of TRPV1 may bind CaM. One putative region was identified in the C-terminal (14), and another was identified in the N-terminal segment (15). We investigated the functional role of CaM in Ca^{2+}-dependent desensitization of capsaicin-activated

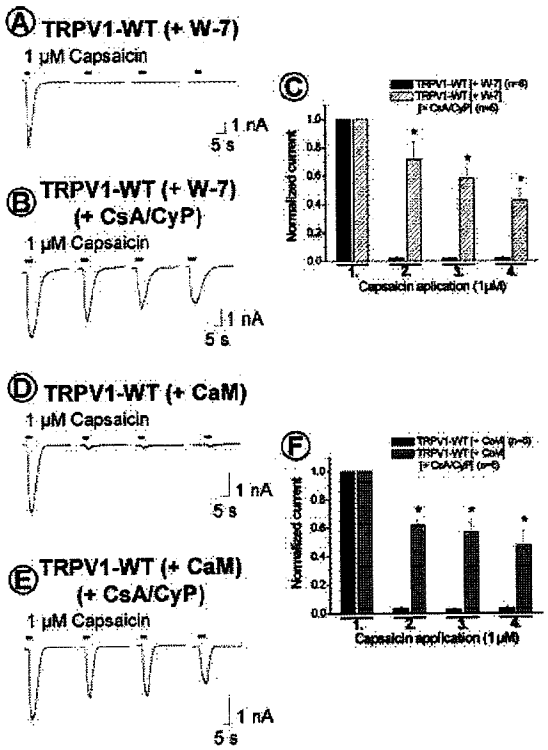

FIG. 3. **Effect W-7, a calmodulin antagonist, and CaM on Ca^{2+}-dependent desensitization of capsaicin-activated TRPV1 currents.** Shown are whole cell current responses of TRPV1 channels to repeated brief (~5-s-long) applications of 1 μM capsaicin in Ca^{2+}-containing (2 mM) bath solution with 100 μM W-7 (*A*) 100 μM W-7 plus 14 nM CsA/17 nM CyP (*B*) 100 μM CaM (*D*) or 100 μM CaM plus 14 nM CsA/17 nM CyP in the pipette solution (*E*). After a whole cell voltage clamp was established, the cells were dialyzed for 10 min before the first capsaicin application. The intervals between capsaicin applications were 2 min. *C* and *F*, The mean amplitudes of currents ± S.E. measured in experiments as described for *A* and *B* or for *D* and *E*, respectively. The amplitudes were normalized to the current amplitude obtained with the first capsaicin application. * indicates a statistically significant difference in the mean amplitudes.

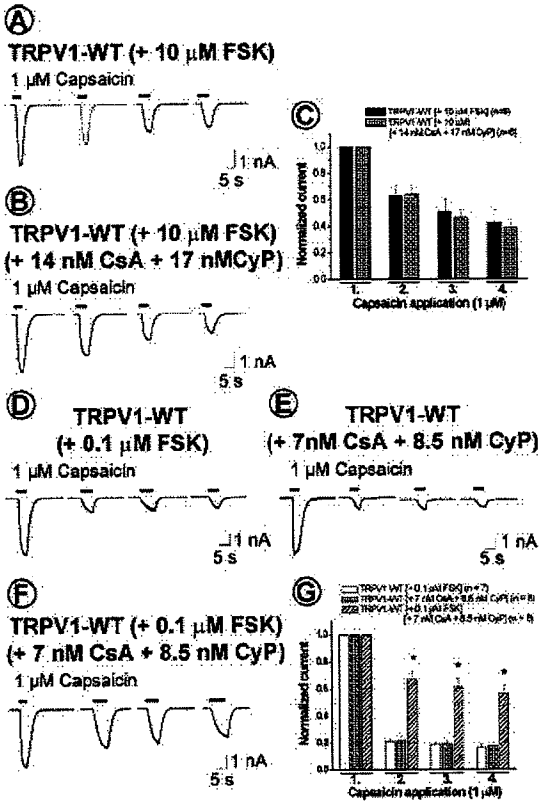

FIG. 4. **Effect of FSK, an indirect activator of protein kinase A, in the absence or presence of CsA complexed to CyP on Ca^{2+}-dependent desensitization of capsaicin-activated TRPV1 currents.** Shown are whole cell current responses of TRPV1-WT channels to repeated brief (~5-s-long) applications of 1 μM capsaicin in Ca^{2+}-containing bath solution after pretreatment for 10 min with 10 μM FSK extracellularly (*A*), 10 μM FSK and the addition of 14 nM CsA + 17 nM CyP to the pipette solution (*B*), 0.1 μM FSK extracellularly (*D*), 7 nM CsA + 8.5 nM CyP in the pipette solution (*E*), or 0.1 μM FSK and the addition of 7 nM CsA + 8.5 nM CyP to the pipette solution (*F*). The intervals between capsaicin applications were 2 min. *C* and *G*, mean amplitudes of currents ± S.E. measured in experiments as described for *A* and *B* and for *D–F*. The amplitudes were normalized to the current amplitude obtained with the first capsaicin application.

TRPV1-WT channels. The experiments were performed as described for those shown in Fig. 1. Pretreatment of cells with 100 μM W-7 in the pipette solution, which is a potent noncompetitive antagonist of calmodulin, did not lead to any change in Ca^{2+}-dependent tachyphylaxis of capsaicin-activated TRPV1-WT currents (Fig. 3, *A* and *C*). W-7 also did not have any significant effect on capsaicin-activated peak currents of TRPV1-WT (Table I). This is in good agreement with an earlier report about the ineffectiveness of W-7 on TRPV1 channel desensitization (14). Tachyphylaxis was significantly decreased when cells were pretreated with 100 μM W-7 along with 14 nM CsA plus 17 nM CyP in the pipette solution (Fig. 3*B*). Here, current amplitudes at the second and fourth capsaicin application were 71.7 ± 12.0 and 42.2 ± 8.1% of that of the first application, respectively (Fig. 3*C*). The decrease in channel tachyphylaxis was comparable with that observed in the presence of CsA plus CyP in the pipette solution (Figs. 1*F* and 3*C*). This indicates that inhibition of calcineurin alone is sufficient to inhibit TRPV1-WT channel desensitization. CaM alone up to concentrations of 100 μM in the pipette solution did not lead to any change in tachyphylaxis of capsaicin-activated TRPV1-WT currents compared with control conditions (Fig. 3, *D* and *F*). Pretreatment of cells with CaM up to concentrations of 100 μM along with 14 nM CsA and 17 nM CyP in the pipette solution led to a significant decrease in tachyphylaxis of capsaicin-activated TRPV1-WT currents (Fig. 3*E*). Here, the results were quantitatively similar to those obtained with CsA·CyP alone or CsA·CyP along with W-7 in the pipette solution (Figs. 1*F* and 3, *C* and *F*).

Phosphorylation by PKA and Dephosphorylation by Calcineurin Regulates Ca^{2+}-dependent Desensitization of TRPV1—In the resting state, TRPV1 is highly phosphorylated, at least when heterologously expressed in CHO-K1 cells (18). PKA is able to phosphorylate TRPV1. However, PKA phosphorylation only becomes obvious in the desensitized state (18). Phosphorylation by PKA partly rescues TRPV1 from desensitization (18, 19). Because inhibition of calcineurin decreases desensitization of capsaicin-activated TRPV1 currents to a similar extent like activation of PKA, we investigated the interplay between PKA activation and calcineurin inhibition and their effect on Ca^{2+}-dependent desensitization of capsaicin-activated TRPV1 currents. As demonstrated previously, pretreatment of cells for 10 min with 10 μM FSK, an activator of adenylate cyclase and thus an indirect PKA activator, led to a significant decrease in channel tachyphylaxis (Fig. 4*A*) (19). Current amplitudes at the second and fourth capsaicin application were 62.7 ± 7.8 and 42.8 ± 9.8% of that at the first application, respectively (Fig. 4*C*). FSK pretreatment did not have any

TABLE II
Effect of various concentrations of FSK and CsA·CyP, applied alone or in combination, on Ca^{2+}-dependent desensitization of capsaicin-activated TRPV1 currents

The experiments were performed as described in the legend to Fig. 4.

FSK treatment	CsA·CyP treatment	Second current[a]	Fourth current[a]	*n*
μM	*nM*	%	%	
0	0	5.1 ± 2.7	3.1 ± 0.4	6
0.1	0	21.5 ± 1.6	16.2 ± 2.1	7
0	1.4/1.7	2.4 ± 0.6	1.1 ± 0.3	5
0.1	1.4/1.7	18.3 ± 1.7	15.7 ± 2.2	3
0.1	0	21.5 ± 1.6	16.2 ± 2.1	7
0	7/8.5	21.9 ± 2.6	17.5 ± 2.4	8
0.1	7/8.5	67.3 ± 5.9	56.0 ± 6.8	8
10	0	62.7 ± 7.8	42.8 ± 9.8	9
0	14/17	58.4 ± 6.9	37.2 ± 6.2	7
10	14/17	64.0 ± 6.8	38.9 ± 6.1	6

[a] The values given for the second and fourth currents are percentages of the values for the first current.

significant effect on capsaicin-activated peak currents in TRPV1-WT (Table I). Pretreatment of cells for 10 min with 14 nM CsA plus 17 nM CyP in the pipette solution along with 10 μM FSK in the external solution did not lead to any significant further decrease in tachyphylaxis compared with that in the presence of 10 μM FSK alone (Fig. 4, *B* and *C*). To exclude the possibility that the decrease in desensitization in the presence of CsA·CyP is caused by PKA phosphorylation caused by disinhibition of PKA by CsA·CyP, we investigated the effect of CsA·CyP on channel tachyphylaxis in the presence of the PKA inhibitor KT5720. In these experiments the decrease in desensitization was similar to that obtained with CsA·CyP alone (data not shown).

To address the question of whether the regulation of desensitization by FSK and CsA·CyP are of an additive or synergistic nature, we also measured the effect of submaximal concentrations of FSK and CsA·CyP on channel tachyphylaxis (Table II). Pretreatment of cells for 10 min with 1.4 nM CsA plus 1.7 nM CyP in the pipette solution did not lead to any significant decrease in tachyphylaxis. With 7 nM CsA plus 8.5 nM CyP in the pipette solution, current amplitudes at the second and fourth capsaicin application were 21.9 ± 2.6 and 17.5 ± 2.4% of that at the first application, respectively (Fig. 4*E*). Comparable effects were obtained when cells were pretreated with 0.1 μM FSK alone (21.5 ± 1.6 and 16.2 ± 2.1%; Fig. 4*D*) or 0.1 μM FSK along with 1.7 nM CsA plus 1.7 nM CyP in the pipette solution (18.3 ± 1.7 and 15.7 ± 2.2%). Pretreatment of cells with 0.1 μM FSK along with 7 nM CsA plus 8.5 nM CyP in the pipette solution, however, led to an effect similar to that obtained with 10 μM FSK along with 14 nM CsA plus 17 nM CyP in the pipette solution (Fig. 4*F*).

These results demonstrate that the concentration dependence for the CsA·CyP-mediated decrease in desensitization is rather steep, as expected for effects that require a cascade of reactions rather than simple one-to-one reactions. Thus, we refrained from performing a more detailed quantitative assessment for the interactions of CsA·CyP with FSK. However, the results obtained by simultaneous application of FSK and CsA·CyP exclude a subadditive action on channel desensitization and are in favor of an additive action for two reasons. First, the effect on tachyphylaxis caused by a submaximal concentration of FSK was unaltered by application of CsA·CyP in a concentration that alone was too weak to effect desensitization. Second, simultaneous application of CsA·CyP and FSK both in concentrations that alone caused a significant but submaximal decrease in channel tachyphylaxis led to a maximal decrease in tachyphylaxis, meaning a decrease that could not be further enhanced by higher concentrations of CsA·CyP or FSK.

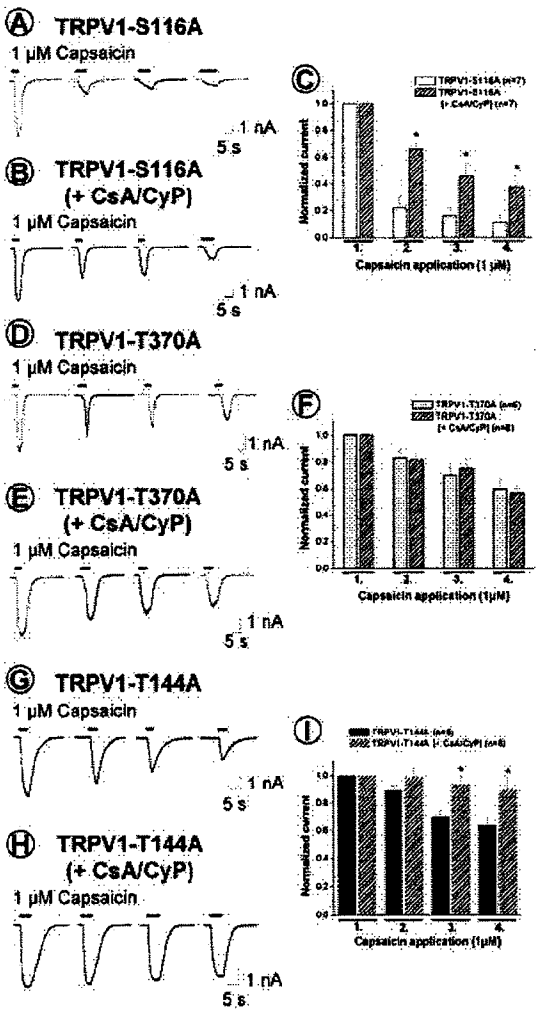

FIG. 5. **Effect of CsA complexed to CyP on Ca^{2+}-dependent desensitization in TRPV1 mutant channels containing the amino acid alanine at putative PKA phosphorylation sites.** Shown are whole cell current responses of mutations S116A (*A* and *B*), T370A (*D* and *E*), and T144A (*G* and *H*) to repeated brief (~5-s-long) applications of 1 μM capsaicin in Ca^{2+}-containing bath solution without (*A*, *D*, and *G*), or with 14 nM CsA + 17 nM CyP in the pipette solution (*B*, *E*, and *H*). After a whole cell voltage clamp was established, the cells were dialyzed for 10 min before the first capsaicin application. The intervals between capsaicin applications were 2 min. *C*, *F*, and *I*, mean amplitudes of currents ± S.E. measured in experiments as described for *A*, *B*, *D*, *E*, *G*, and *H*. The amplitudes were normalized to the current amplitude obtained with the first capsaicin application. * indicates a statistically significant difference between the mean values obtained without and with CsA·CyP treatment.

Among several putative PKA phosphorylation sites of TRPV1, amino acid residues Ser^{116} and Thr^{370} seem to be the most critical ones for PKA-dependent modulation of TRPV1 (18, 19). Substitution of Ser^{116} or Thr^{370} with either alanine or aspartate led to mutant channels that could not be modulated by PKA (19). We now investigated the effect of calcineurin inhibition on Ca^{2+}-dependent desensitization in TRPV1 mutant channels S116A and T370A. Experiments were performed as described for those shown in Fig. 1. Under control conditions, TRPV1-S116A clearly showed some tachyphylaxis that was however less pronounced compared with TRPV1-WT (Fig. 5*A*). Here, the current amplitudes at the second and fourth capsaicin application were 22.6 ± 8.7 and 11.0 ± 4.0% of that of the first application, respectively, and were significantly larger than those for TRPV1-WT under

control conditions (Figs. 1*F* and 5*C*). Pretreatment of TRPV1-S116A for 10 min with 14 nM CsA and 17 nM CyP in the pipette solution, however, led to a significant decrease in tachyphylaxis compared with control conditions (Fig. 5*B*). Current amplitudes at the second and fourth capsaicin application were 66.1 ± 4.1 and 38.2 ± 7.9% of that of the first application, respectively (Fig. 5*C*). This indicates that dephosphorylation by calcineurin of a residue other than Ser^{116} might significantly contribute to desensitization.

As described before (19), TRPV1-T370A is one of the least desensitizing alanine mutation at putative PKA phosphorylation sites under control conditions (Fig. 5*D*). Here, current amplitudes at the second and fourth capsaicin application were 83.2 ± 5.4 and 59.5 ± 7.9% of that of the first application (Figs. 1*F* and 5*F*). Pretreatment for 10 min with 14 nM CsA and 17 nM CyP in the pipette solution, however, had no statistically significant effect on channel tachyphylaxis (Fig. 5*E*). Here, current amplitudes at the second and fourth capsaicin application were 82.1 ± 3.1 and 56.7 ± 5.5% of that of the first application, respectively (Fig. 5*F*). Because mutation T370A constitutively shows only weak tachyphylaxis, it could be speculated that inhibition of calcineurin will not produce any measurable effect on tachyphylaxis, even if the channel was a substrate for calcineurin. To exclude this possibility, we investigated the effect of calcineurin inhibition on mutation T144A, which is another channel exhibiting impaired tachyphylaxis (19). In this mutation, pretreatment for 10 min with 14 nM CsA and 17 nM CyP in the pipette solution significantly decreased tachyphylaxis compared with control conditions (Fig. 5, *G–I*). Pretreatment of cells with CsA·CyP did not have any significant effect on the peak amplitudes of capsaicin-activated currents in any of these mutant channels (Table I). These observations support the idea that amino acid residue Thr^{370} might be a key site for calcineurin-induced dephosphorylation of TRPV1.

Phosphorylation by PKCε Does Not Modulate Ca^{2+}-dependent Desensitization of Capsaicin-activated TRPV1 Currents—In addition to PKA, the ε-isoform of PKC was demonstrated to directly modify TRPV1 and to sensitize heat- and capsaicin-activated currents (20–22). Here, residues Ser^{502} and Ser^{800} were suggested to be the major substrates for PKC-dependent phosphorylation (22). However, there is no evidence for a PKC-dependent modulation of TRPV1 desensitization so far (19). We were interested in whether or not there is any interplay between PKC phosphorylation and calcineurin-modulation of TRPV1. Experiments were performed as described for those shown in Fig. 1.

As demonstrated before, pretreatment of cells with PMA (0.1 μM), an activator of PKC, did not have any effect on channel tachyphylaxis (Fig. 6*A*). PMA pretreatment also did not have any significant effect on the peak amplitudes of capsaicin-activated currents (Table I). Pretreatment for 10 min with 14 nM CsA and 17 nM CyP in the pipette solution along with 0.1 μM PMA in external buffer significantly decreased channel tachyphylaxis (Fig. 6, *B* and *C*).

Calcineurin inhibition by CsA·CyP also significantly decreased tachyphylaxis in double mutation S502A/S800A, in which putative PKC phosphorylation sites were disrupted (Fig. 6, *D*, *E*, and *G*). In the same double mutation S502A/S800A, FSK decreased tachyphylaxis to a similar extent like CsA·CyP (Fig. 6, *F* and *G*). Pretreatment of cells with CsA·CyP or FSK did not have any significant effect on peak amplitudes of capsaicin-activated currents in mutation S502A/S800A (Table I). These results confirm that phosphorylation of TRPV1 by PKCε indeed is not involved in the channel desensitization process.

Activation of PKA Increases Capsaicin Sensitivity of TRPV1—To determine the effect of calcineurin inhibition by

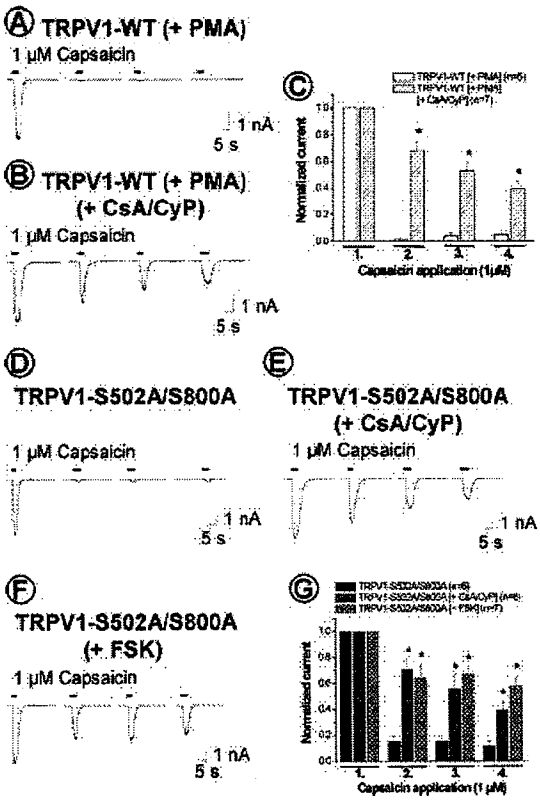

FIG. 6. **The role of putative PKC phosphorylation sites on Ca^{2+}-dependent desensitization of capsaicin-activated TRPV1 currents.** *A* and *B*, whole cell current responses of TRPV1-WT channels to repeated brief (~5 s long) applications of 1 μM capsaicin in Ca^{2+}-containing bath solution after pretreatment for 10 min with 0.1 μM PMA extracellularly (*A*) or 0.1 μM PMA and the addition of 14 nM CsA + 17 nM CyP to the pipette solution (*B*). The intervals between capsaicin applications were 2 min. *C*, mean amplitudes of currents ± S.E. measured in experiments as described for *A* and *B*. The amplitudes were normalized to the current amplitude obtained with the first capsaicin application. * indicates a statistically significant difference between the mean values. *D–F*, whole cell current responses of the double mutation TRPV1-S502A/S800A to repeated brief (~5 s long) applications of 1 μM capsaicin in Ca^{2+}-containing bath solution without (*D*) or with 14 nM CsA + 17 nM CyP in the pipette solution (*E*) or after pretreatment for 10 min with of 10 μM FSK extracellularly (*F*). The intervals between capsaicin applications were 2 min. *G*, mean amplitudes of currents ± S.E. measured in experiments as described for *D–F*. The amplitudes were normalized to the current amplitude obtained with the first capsaicin application. * indicates a statistically significant difference between the mean values compared with control.

CsA·CyP and PKA activation by FSK on the sensitivity of TRPV1-WT, TRPV1-S116A, and TRPV1-T370A toward capsaicin, we measured the concentration dependence of capsaicin responses in Ca^{2+}-free bath solutions and determined the half-maximal activating concentrations (EC_{50}) before and after pretreatment with 10 μM FSK in the external solution or 14 nM CsA with 17 nM CyP in the pipette solution. Under control conditions, EC_{50} for TRPV1-WT was 242 ± 2 nM, the Hill coefficient (h) was 1.9 ± 0.1 (Fig. 7*A*). These values are in reasonable agreement with values found previously for TRPV1 expressed in HEK293t cells (19). The capsaicin concentration-response curve was significantly shifted leftward by ~4.5-fold after pretreatment with FSK (EC_{50} = 52 ± 2 nM; h = 1.8 ± 0.1; Fig. 7*A*) but was not significantly changed after pretreatment with CsA·CyP (EC_{50} = 190 ± 11 nM; h = 1.8 ± 0.2; Fig. 7*A*).

These results confirm earlier reports that the PKA pathway not only regulates desensitization of TRPV1 but also sensitizes

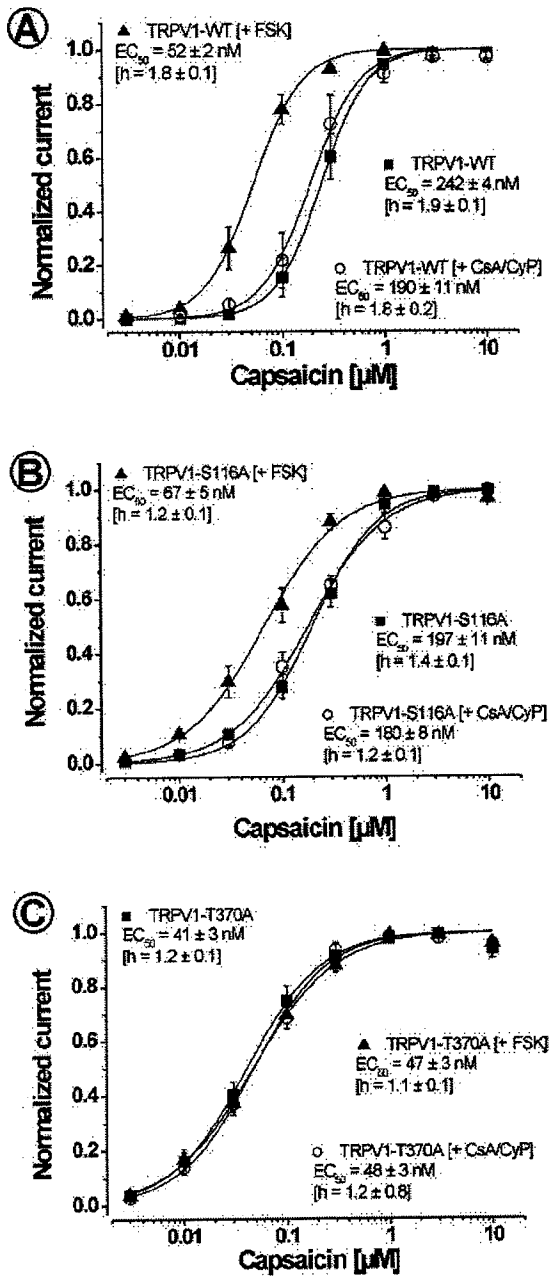

FIG. 7. **Concentration dependence of capsaicin responses in TRPV1-WT channels (*A*) and mutations TRPV1-S116A (*B*) and TRPV1-T370A (*C*) in the absence or presence of FSK or CsA·CyP.** Increasing capsaicin concentrations were applied to cells expressing TRPV1-WT or mutant channels in Ca^{2+}-free external bath solution in the absence (■; $n = 8$ for WT, $n = 6$ for S116A, and $n = 5$ for T370A) or presence of 10 μM FSK in the external solution (▲; $n = 10$ for WT, $n = 6$ for S116A, and $n = 6$ for T370A) or 14 nM CsA + 17 nM CyP in the pipette solution (○; $n = 6$ each for WT, S116A, and T370A). The intervals between applications were 1 min. The peak amplitudes of capsaicin-activated currents were measured, normalized to the maximum response measured in each cell, and plotted against the capsaicin concentration. The *lines* represent the fits of the data to the Hill equation. EC_{50} values and Hill coefficients (h) are given in the figure.

the channel to capsaicin. These results also imply that under resting conditions, the channel is only partly phosphorylated at PKA sites, unlike suggested earlier (18).

For TRPV1-S116A, the EC_{50} was 197 ± 11 nM under control conditions, and the Hill coefficient (h) was 1.4 ± 0.1 (Fig. 7*B*). These values are in reasonable agreement with values found previously for TRPV1-S116A (19). Comparable with WT, in this mutation the capsaicin concentration-response curve was significantly shifted leftward by ~3-fold after pretreatment with FSK (EC_{50} = 67 ± 5 nM; h = 1.2 ± 0.1; Fig. 7*B*) but was not significantly changed after pretreatment with CsA·CyP (EC_{50} = 180 ± 8 nM; h = 1.2 ± 0.1; Fig. 7*B*). Earlier, we have demonstrated that in mutation TRPV1-S116, there was a slight reduction in tachyphylaxis after pretreatment with FSK, which, however, was not statistically significant (19). The data in this study confirm that one or several other residues in addition to Ser^{116} are very likely involved in the mechanism of PKA-dependent modulation of TRPV1.

For TRPV1-T370A, sensitivity toward capsaicin was significantly lower under control conditions compared with WT and TRPV1-S116A (EC_{50} = 41 ± 3 nM; h = 1.2 ± 0.1; Fig. 7*C*). In this mutation, pretreatment with FSK or CsA·CyP did not have any significant effect on the EC_{50} values or Hill coefficients. These observations support the idea that amino acid residue Thr^{370} might be a key site for PKA-mediated phosphorylation of TRPV1. Concentration-effect experiments were performed in Ca^{2+}-free solutions to prevent channel desensitization. This way, the ineffectiveness of calcineurin in these experiments can be explained.

Inhibition of Calcineurin Decreases Ca^{2+}-dependent Desensitization of Proton-activated TRPV1-WT Currents—TRPV1 is a multimodal sensor that in addition to vanilloids is also activated by protons and heat (1, 2), anandamide (3), ethanol (5), various lipoxygenase products, and other lipids related to arachidonic acid (4). Although not unequivocal, activation of TRPV1 by protons has been demonstrated to lead to channel desensitization in a Ca^{2+}-dependent manner (11). This desensitization can be partly rescued by PKA activation as well (18).

We studied the effect of calcineurin inhibition on desensitization of proton-activated TRPV1 currents. As HEK293t cells were shown to endogenously express an acid sensing ion channel (hASIC1a) (27), channels were transiently expressed in HeLa cells for these experiments.

Under control conditions, TRPV1-WT showed pronounced proton-induced tachyphylaxis (Fig. 8, *A* and *C*) that was qualitatively similar to capsaicin-induced tachyphylaxis. Proton-induced tachyphylaxis was significantly decreased when cells were pretreated for 10 min with 14 nM CsA plus 17 nM CyP in the pipette solution (Fig. 8, *B* and *C*). Similarly, CsA·CyP decreased acute desensitization of TRPV1-WT induced by prolonged application (30 s) of protons (Fig. 8, *D–F*). Peak amplitude of proton-activated currents were unaffected by CsA·CyP (Table I).

DISCUSSION

In this study, we show that specific inhibition of calcineurin (protein phosphatase 2B) significantly decreases Ca^{2+}-dependent desensitization of capsaicin- and proton-activated TRPV1 currents. This effect is qualitatively and quantitatively similar to but independent from that obtained by extracellular application of FSK and cannot be further enhanced by simultaneous application of FSK and CsA·CyP. In mutation T370A, but not mutations S116A and T144A, desensitization properties are unaffected by calcineurin inhibition. In double mutation S502A/S800A, in which putative PKC phosphorylation sites are disrupted, both calcineurin inhibition and PKA activation decrease desensitization of capsaicin-activated currents similar to WT channels. We conclude that Ca^{2+}-dependent desensitization of TRPV1 might be in part regulated through channel dephosphorylation by calcineurin and channel phosphorylation by PKA, possibly involving Thr^{370} as a key amino acid residue.

Dephosphorylation and Desensitization—Protein phosphorylation and dephosphorylation is a major mechanism in mam-

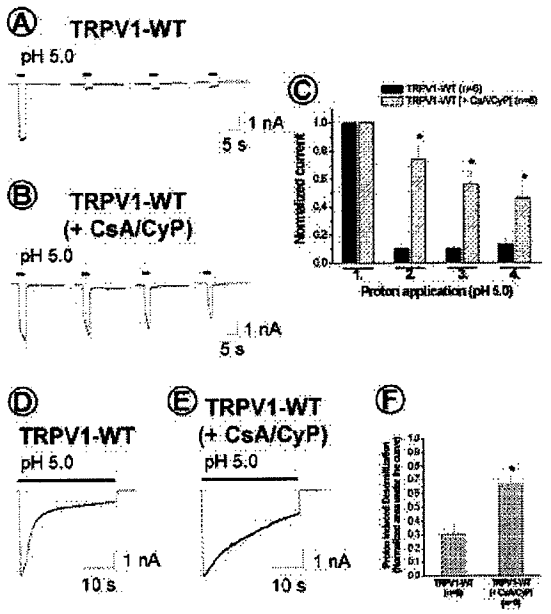

FIG. 8. **Effect of CsA and CyP on Ca^{2+}-dependent desensitization of proton-activated TRPV1-WT currents.** Shown are whole cell current responses of TRPV1 channels to repeated brief (~5 s long) applications of protons (pH 5.0) in Ca^{2+}-containing (2 mM) bath solution without (*A*) or with 14 nM CsA + 17 nM CyP in the pipette solution (*B*). After a whole cell voltage clamp was established, the cells were dialyzed for 10 min before the first capsaicin application. The intervals between proton applications were 2 min. *C*, mean amplitudes of currents ± S.E. measured in experiments as described for *A* and *B*. The amplitudes were normalized to the current amplitude obtained with the first proton application. * indicates a statistically significant difference in the mean amplitude compared with control. *D* and *E*, whole cell current responses of TRPV1 channels evoked by a 30-s-long application of protons (pH 5.0) in Ca^{2+}-containing (2 mM) bath solution without (*D*) and with 14 nM CsA + 17 nM CyP in the pipette solution (*E*). *F*, the areas under the current curves were measured and normalized to an idealized, nondesensitizing current of respective size. The *bars* represent the mean values ± S.E. * indicates a statistically significant difference compared with control.

malian cells for regulating structure and function and responding to external stimuli. In a previous study, ^{32}P labeling and immunoprecipitation revealed that TRPV1 is highly phosphorylated in the resting state, at least when heterologously expressed in CHO-K1 cells, and that phosphorylation could be significantly reduced by application of a desensitizing concentration of capsaicin (18). In another study, dephosphorylation could be prevented by coapplication of capsaicin and FK-506, another calcineurin inhibitor. Moreover, evidence was presented that phosphorylation of TRPV1, presumably at a CaMKII consensus phosphorylation site (Ser^{502}/Thr^{704}), is a prerequisite for the capsaicin binding capacity of TRPV1 (13).

The decrease in desensitization by the CsA·CyP complex found in this study confirms previously reported results obtained in rat dorsal root ganglion neurons (12) and strongly suggests that dephosphorylation by calcineurin indeed comprises channel activity and thus elicits desensitization. Because calcineurin is a Ca^{2+}- and calmodulin-dependent phosphatase, this mechanism could account for the Ca^{2+} dependence of desensitization. Similar mechanisms have been suggested to underlie the desensitization of the ionotropic receptor P2X3 (28), the neuronal nicotinic acetylcholine receptor (29) and the *N*-methyl-D-aspartate receptor (30, 31). Interestingly, CsA alone had no effect on desensitization, indicating that HEK293t cells might not contain sufficient cyclophilin A to allow formation of the inhibitory complex, at least not under our experimental conditions.

Calcineurin inhibition not only decreased channel tachyphylaxis but also decreased acute desensitization elicited by a 30-s-long capsaicin application, an effect that could not be observed with PKA activation in an earlier study (19). This disparity in effects between calcineurin inhibition and PKA activation suggests that dephosphorylation might be a faster process as compared with rephosphorylation. Moreover, dephosphorylation might not require a closed or ligand-free channel, as hypothesized for the process of rephosphorylation.

Calcineurin inhibition also decreased desensitization of proton-activated TRPV1 currents under our experimental conditions. Unlike suggested by others (13), we conclude that activation by capsaicin and activation by protons most probably initiate comparable mechanisms of Ca^{2+}-dependent desensitization.

The Role of CaM—Calcineurin acts in a Ca^{2+}- and calmodulin-dependent manner. CaM is a dominant Ca^{2+} sensor for Ca^{2+}-dependent inactivation in many ion channels (24–26). There is accumulating evidence that multiple regions of TRPV1 may bind CaM. One putative region was identified in the C-terminal (14), and another was identified in the N-terminal segment (15).

In our experiments both CaM and the calmodulin antagonist W-7 did not have any effect on TRPV1 desensitization or peak amplitudes of capsaicin-activated currents. The ineffectiveness of the CaM inhibitor is in good agreement with an earlier report (14) and might suggest that CaM is not involved in Ca^{2+}-dependent desensitization of capsaicin-activated TRPV1 currents. However, CaM inhibitors would only be expected to inhibit desensitization if CaM acted as a free molecule (32, 33). This, however, might not be the case as suggested by recent reports (15, 22).

CaM was demonstrated to mediate Ca^{2+} inhibition of TRPV1 in inside-out excised patches of *Xenopus* oocytes and HEK293 cells expressing TRPV1. In that study, CaM was applied to the intracellular site of the channel together with 50 μM free Ca^{2+} (15). We cannot exclude that the ineffectiveness of CaM in our study is due to the rather uncontrolled intracellular free Ca^{2+} concentration under our experimental conditions. Thus, from our data, we cannot derive profound evidence for or against a role of CaM in TRPV1-WT channel desensitization.

Functional Coupling of Calcineurin and PKA—The decrease in desensitization of capsaicin-activated currents by calcineurin inhibition in this study was qualitatively and quantitatively similar to that obtained by PKA activation. Simultaneous application of FSK and CsA·CyP in submaximal and maximal concentrations suggest additive actions of FSK and CsA·CyP rather than subadditive or synergistic actions. In mutation T370A, in which a putative PKA phosphorylation site is disrupted, desensitization properties were unaffected by calcineurin inhibition. Calcineurin inhibition, however, did reduce desensitization in both mutations S116A, in which another critical PKA phosphorylation site is disrupted (18, 19), and mutation T144A, which shares similar desensitization properties with mutation T370A (19). These results suggest that Ca^{2+}-dependent desensitization of TRPV1 might be in part regulated through channel dephosphorylation by calcineurin and channel phosphorylation by PKA possibly involving Thr^{370} as a key amino acid residue. A functional coupling of calcineurin and PKA was proposed before in mouse ventricular myocytes to control Ca^{2+} influx through Ca^{2+} channels and Ca^{2+} release through ryanodine receptors (34). In these cells, immunofluorescence also revealed colocalization of calcineurin and PKA.

We hypothesize that a similar mechanism could control excitability of nociceptive sensory neurons by regulating desensitization and thus channel availability. The interplay between

CaMKII and PKA remains to be characterized in future studies.

TRPV1 channel phosphorylation by PKA (16) and PKC (22) to control activation thresholds and TRPV1 channel phosphorylation/dephosphorylation by PKA (18, 19), CaMKII (13), and calcineurin to regulate desensitization/availability might allow fine tuning of the nociceptor in response to a noxious environment.

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BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

Novel Non-vanilloid VR1 Antagonist of High Analgesic Effects and Its Structural Requirement for VR1 Antagonistic Effects

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Abstract—A novel non-vanilloid VR1 antagonist consisting of a new vanilloid equivalent exhibits excellent analgesic effects as well as highly potent antagonistic activities in both capsaicin single channel and calcium uptake assays. In addition, the structural requirement for the vanilloid equivalent of the potent VR1 antagonist has also been elucidated.

Introduction

Over the past few years, the amount of information from studies on pain transmission by capsaicin has dramatically increased, revealing novel targets for the advent of new pain therapies. Recently, a giant step forward came with the identification of a protein called the vanilloid receptor 1 (VR1). VR1 can be activated not only by vanilloid ligands including capsaicin and endocannabinoid, but also by noxious heat (>43 °C) and protons (extracelluar pH<6). Particularly, vanilloids and low pH are known to reduce the temperature threshold for VR1 activation.[1–3] Our recent studies have also revealed several 12-lipoxygenase metabolites of arachidonic acid, including 12-(*S*)-HPETE, as endogenous activators of the neuronal vanilloid receptor.[4]

Since capsaicin was found as an excellent vanilloid receptor agonist, considerable efforts toward the development of a novel analgesic have been continued. However, the small therapeutic window between these effects and the excitatory side effects, such as hyperthermia, bronchoconstriction, increased GI mobility, and hypertension, precluded the development of capsaicin as a systemic agent. Thus, recent studies on VR1 agonists or antagonists have focused on separating the excitatory effects of capsaicin analogues from the antinociceptive properties of these molecules.

In particular, the idea that VR1 functions as an integrator of multiple pain-producing stimuli implies that VR1 antagonists or channel blockers should have profound antinociceptive effects,[5] especially in inflammatory pain models.[6] Many research groups, including ours,[7] are engaged in developing potent and novel VR1 antagonists, although the therapeutically useful antagonists are not currently available.[2,8,9]

On the basis of the previous studies on vanilloid receptor agonists and antagonists, as well as our recent exciting findings,[4] we have looked for the non-vanilloid VR1 antagonists by developing the ideal vanilloid equivalents, which might provide the perfect analgesic effects without the side effects caused by vanilloid receptor agonists. Consequently, we have developed an excellent VR1 antagonist (SC0030) and revealed a part of the mechanistic aspect of SC0030.[10] We herein report the results of our recent studies on SC0030 from the view-

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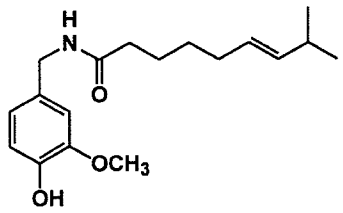
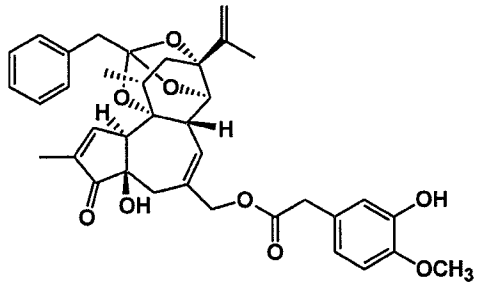
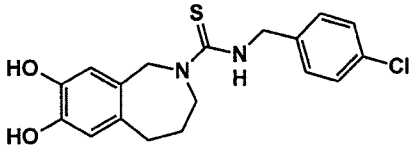


Capsaicin



Resiniferatoxin



Capsazepine

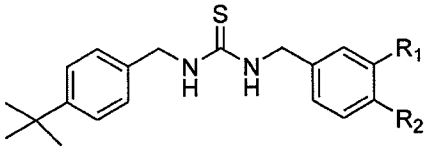
point of the minimal structural requirement for its potent VR1 antagonistic effects as well as its therapeutic application.

Since our initial work was focused on the development of novel vanilloid equivalents, which function as both hydrogen bonding donors and/or acceptors, like the vanilloid moiety of capsaicin, a large number of vanilloid equivalents have been designed and synthesized in our laboratory. The representative analogues, shown in Table 1, were selected for the comparison of their VR1 antagonistic effects because they have the characteristic functions of the vanilloid equivalent.

Chemistry

The advanced synthesis of SC0030[10] is outlined in Scheme 1. Cyanation of the methansulfonamide **2**, prepared by mesylation of the commercially available 2-fluoro-4-iodoaniline, with CuCN at 130 °C provided the cyano intermediate **3** in 75% of two steps yield. Conversion of the nitrile **3** to the benzylamine **4** was carried out by BH_3 reduction. Finally, the coupling of the amine salt **4** with 4-*t*-butylbenzyl isothiocyanate afforded the thiourea **5** (SC0030) in 75% of two steps yield.

Table 1. Ca^{2+} uptake inhibition of **SC0030** and its representative structural analogues in DRG neurons



Analogues	R_1	R_2	IC_{50} (μM)
5 (SC0030)	F	$NHSO_2CH_3$	0.037
10	$NHSO_2CH_3$	F	24.30
14 (MK056)	H	$NHSO_2CH_3$	0.110
17	F	H	>30
18	H	H	Not active
	Capsazepine		0.59

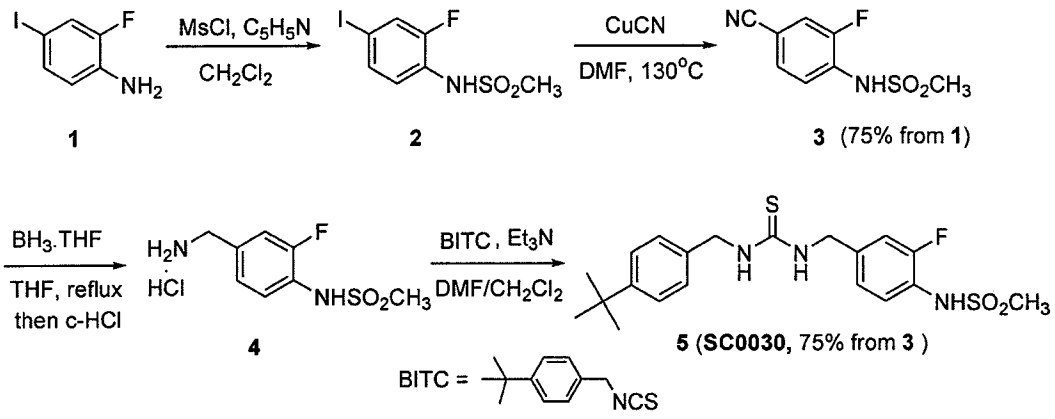
The analogue **10** was synthesized from the commercially available 4-fluoro-3-nitrobenzaldehyde **6**. Reaction of benzaldehyde **6** with hydroxylamine and reduction of the resulting oxime followed by Boc-protection provided the Boc-protected benzylamine **7**. Bismesylation of **7** and Boc-deprotection followed by coupling of the benzylamine salt with 4-*t*-butylbenzyl isothiocyanate afforded the thiourea **9** in 67% yield. Finally, selective removal of one mesyl group of **9** by NaOH treatment afforded the desired thiourea **10** in 54% yield (Scheme 2). The analogue **14** (MK056) was synthesized from *p*-nitrobenzylamine **11** by a five steps sequence. The sulfonamide intermediate **13** was prepared by Boc-protection of nitrobenzylamine **11**, catalytic reduction of nitro group, followed by mesylation of the resulting benzylamine. The sulfonamide **13** was converted to the analogue **14** by the procedure previously described[10] (Scheme 3). Analogues **17** and **18** were conveniently prepared by coupling of the corresponding benzylamine with 4-*t*-butylbenzyl isothiocyanate (Scheme 4).

Biological Assays

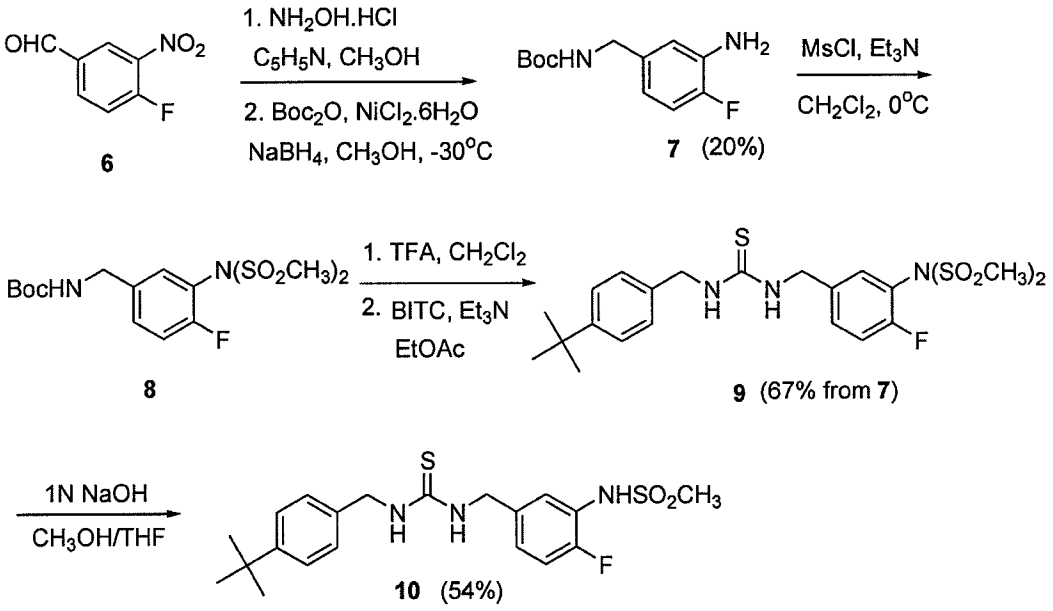
Dorsal root ganglion (DRG) neurons were prepared from neonatal Sprague–Dawley rats by a modification of the previously described method, and $^{45}Ca^{2+}$ uptake experiments were carried out by the reported procedure.[11] The IC_{50} is expressed as the concentration of the tested compound required to reduce the response to 0.5 μM capsaicin by 50%.

In order to determine whether the tested compounds activate or antagonize the vanilloid receptor, we recorded the single-channel current of the vanilloid receptor in inside-out membrane patches isolated from cultured sensory neurons.[4]

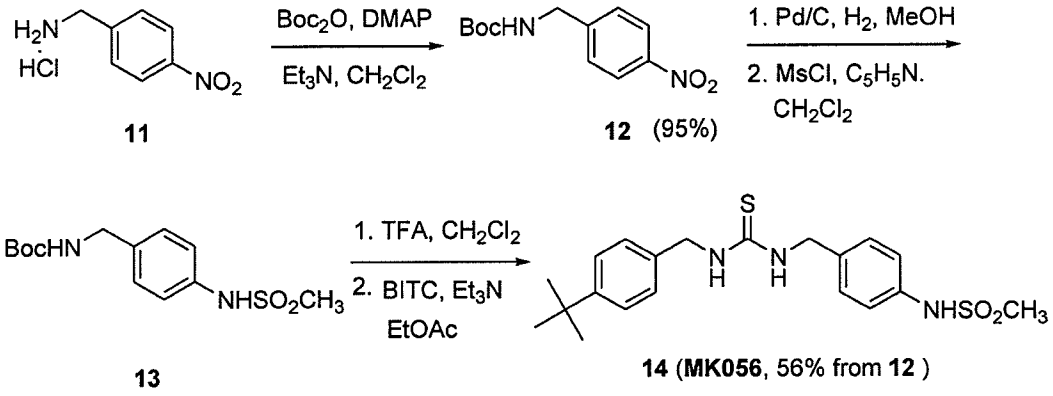
The PBQ-induced writhing assay carried out with male mice, which received an intraperitoneal injection of the chemical irritant phenyl-*p*-quinone.[12] A reduction in the number of writhes responding to phenyl-*p*-quinone relative to the number responding in the saline control



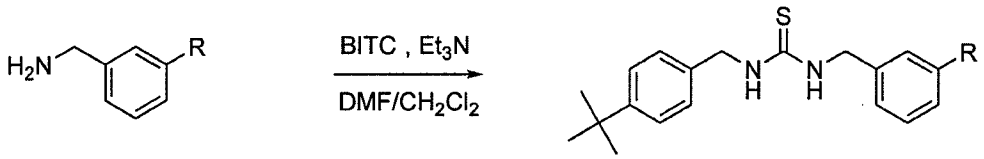
Scheme 1. Synthesis of analogue **5** (SC0030).



Scheme 2. Synthesis of analogue **10**.



Scheme 3. Synthesis of analogue **14** (MK056).



15 R = F

16 R = H

17 R = F (70%)

18 R = H (98%)

Scheme 4. Synthesis of analogues **17** and **18**.

group was considered to be indicative of an antinociceptive effect.

Results and Discussion

We initially examined the in vitro activities of more than hundred synthetic compounds, which were designed based on the structures of the reported natural and unnatural agonists and antagonists. The analogue SC0030 was finally selected as the best antagonist based on the in vitro assays employing calcium uptake of the vanilloid receptor. On the structural basis of the synthesized ligands,[13] the structural requirements of the vanilloid equivalents for the potent VR1 antagonistic effects were analyzed from Table 1.

Fluoride for R_1, which displaces methoxy of the vanilloid moiety of capsaicin turned out to be the best substituent as a H-bonding acceptor while methansufonamide for R_2, which displaces hydroxy of the vanilloid moiety was the best substituent as a H-bonding donor. In particular, SC0030, which possesses both fluoro and methansulfonamido substituents was 16-fold more potent than capsazepine in Ca^{2+} uptake inhibition, as a known VR1 antagonist. This novel ligand deserves great attention as a potent VR antagonist with an IC_{50} of 0.037 μM in Ca^{2+} uptake inhibition test, which makes it one of the most potent non-vanilloid VR antagonistic ligands described to date. The analogues possessing other substituents for R_1 and/or R_2 provided lower antagonistic effects or agonistic effects. Switch of fluoro (R_1) and methansulfonamido (R_2) groups significantly drops Ca^{2+} uptake inhibition activity. Elimination of fluoro group for R_1 (**14**) decreases the antagonistic activity. However, the analogue **14** still exhibits the higher potency than capsazepine. The analogue **17**, which possesses only fluoro substituent for R_1 retains antagonistic activity although the potency is quite low. However, the analogue **18**, which possesses neither fluoro nor methansulfonamido substituent, completely loses the antagonistic activity. In conclusion, the fluoro and methansulfonamido groups for R_1 and R_2, respectively, are obviously essential for the high Ca^{2+} uptake inhibition activity of the vanilloid equivalent. The fluoro substituent itself provides weak antagonistic activity. However, it is crucial for the high antagonistic activity of the vanilloid equivalents.

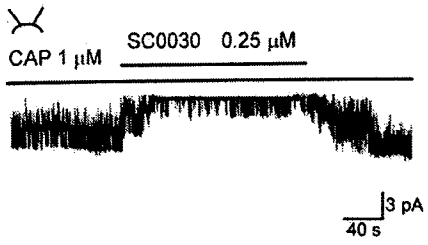


Figure 1. Reversible antagonistic effects of SC0030 on the capsaicin receptor.

Consistent with observation of calcium uptake results, the VR1 antagonism of SC0030 was confirmed by inhibition of the capsaicin-induced action on patch-clamped rat DRG neurons. As shown in Figure 1, the application of 1 μM of capsaicin greatly activates capsaicin receptors in inside-out membrane patches. However, SC0030 inhibited the channel activity (85.7±2.4% reduction, $n=4$) evoked by capsaicin when 0.25 μM of SC0030 was applied to the patch together with 1 μM of capsaicin. After the SC0030 was removed, the application of 1 μM of capsaicin again activated the channel activity. Thus, these results suggest that 0.25 μM SC0030 clearly antagonizes the action of capsaicin at the capsaicin receptor in a reversible manner. Figure 2 shows the magnitude of inhibition by 0.25 μM of SC0030 was comparable to the magnitude obtained after the application of 10 μM of capsazepine.

The analgesic effect of SC0030 was evaluated from the viewpoint of the therapeutic applications and it was confirmed by the PBQ-induced writhing antinociceptive assay. The results presented in Figure 3 show that

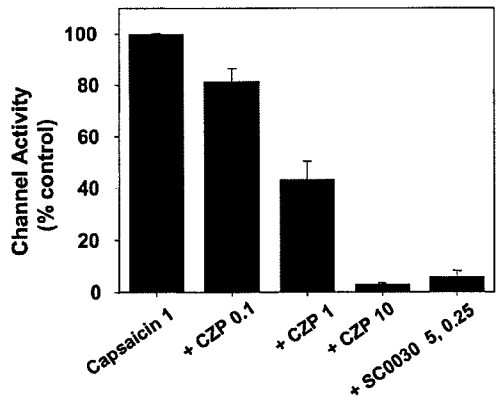


Figure 2. Comparison of the antagonistic effects of SC0030 and capsazepine on capsaicin receptor activity in sensory neurons. Numbers represent concentrations in μM.

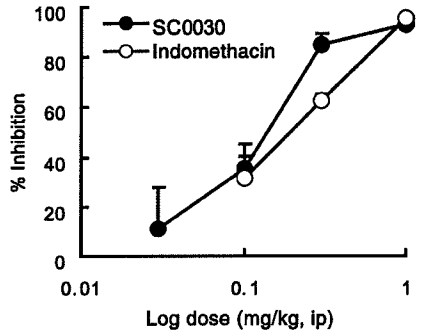


Figure 3. Potent and dose-dependent analgesic effect of SC0030 on PBQ-induced writhing in mice ($n=10$/group). Values (mean ± SEM) represent % inhibition of writhing responses.

SC0030 inhibited the writhing in a dose-dependent manner, and was almost equipotent or slightly more potent than indomethacin, a nonselective COX inhibitor.

For further confirmation of the therapeutic uses of SC0030, we examined the tests related to the pungency, which generally arises from the agonistic effects of capsaicin analogues. We performed a capsaicin-induced licking test,[14] on the basis that the capsaicin-evoked activation of sensory afferent neuron is associated with the activation of selective membrane vanilloid receptors. SC0030 dose-dependently inhibited capsaicin-induced nociception as well as pain-related behavioral responses. In addition, SC0030 did not cause the protective eye-wiping movement in the rat upon intraocular instillation. The topical application of the capsaicin to the eye of experimental animals is known to evoke immediate pain, as revealed by the increased number of scratching movements toward the treated eye.

As mentioned earlier, the previous studies on VR1 agonists reported that systemic and intrahypothalamic injection of capsaicin produce hypothermia. The VR1 expressing neurons in the brain area are thought to play an important role in the central control of thermoregulation. As we anticipated, no change of body temperature by the administration of SC0030 in the rat was observed.

In summary, in the present work we have elucidated the structural requirement for the potent and selective VR1 antagonist, particularly for the vanilloid equivalent. The synthetic SC0030 acts as a strong inhibitor of Ca^{2+} uptake, with a much lower IC_{50} value than that of capsazepine. In addition, it displays a potent analgesic activity similar to that of indomethacin in vivo. Moreover, this compound is devoid of the important shortcomings of capsaicin, such as hypothermia and pungency. Most importantly, SC0030 can be conveniently synthesized on more than a hundred-gram scale to ensure a substantial quantity. This novel VR1 antagonist would be highly useful not only as a new candidate for non-vanilloid analgesic development but also as a tool to investigate the VR1-mediated pain response.

Acknowledgements

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EXHIBIT A

[^{3}H]DAMGO (Amersham)[18]. Measurements were done in triplicate. Binding data was analysed using the LIGAND program (supplied by P. Munson).

In situ hybridization

Cryostat sections (13 μm) of fresh brain tissue were hybridized with ^{35}S-labelled oligonucleotide probes specific for the dopamine D1 and D2 receptors[1] (see Supplementary Information).

Immunohistochemistry

Mice were anaesthetized with sodium pentabarbital and fixed transcardially with 4% paraformaldehyde in 0.1 M phosphate buffer, the brains removed and sections cut at 40 μm for processing with antibodies against the mu opiate receptor (Chemicon), c-Fos or Fos-B (Santa Cruz). The Fos-B antibody recognizes both full-length and truncated products of the gene. To study Fos-B expression, mice were treated daily with two injections of morphine in increasing doses (20, 40, 60, 80 mg kg^{-1}). On the last day, a single dose of 100 mg kg^{-1} was given in the morning and mice were perfused 6 h later.

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Vanilloid receptor-1 is essential for inflammatory thermal hyperalgesia

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The vanilloid receptor-1 (VR1) is a ligand-gated, non-selective cation channel expressed predominantly by sensory neurons. VR1 responds to noxious stimuli including capsaicin, the pungent component of chilli peppers, heat and extracellular acidification, and it is able to integrate simultaneous exposure to these stimuli[1,2]. These findings and research linking capsaicin with nociceptive behaviours (that is, responses to painful stimuli in animals[3] have led to VR1 being considered as important for pain sensation. Here we have disrupted the mouse VR1 gene using standard gene targeting techniques. Small diameter dorsal root ganglion neurons isolated from VR1-null mice lacked many of the capsaicin-, acid- and heat-gated responses that have been previously well characterized in small diameter dorsal root ganglion neurons from various species. Furthermore, although the VR1-null mice appeared normal in a wide range of behavioural tests, including responses to acute noxious thermal stimuli, their ability to develop carrageenan-induced thermal hyperalgesia was completely absent. We conclude that VR1 is required for inflammatory sensitization to noxious thermal stimuli but also that alternative mechanisms are sufficient for normal sensation of noxious heat.

We have used homologous recombination in embryonic stem (ES) cells to generate a mouse lacking transmembrane domains 2–4 of the mVR1 gene (Fig. 1a). Germline chimaeras were crossed onto C57Bl/6J females to generate heterozygotes, which were intercrossed giving rise to overtly healthy homozygous mutant offspring in the expected mendelian ratio (average litter numbers: VR1$^{-/-}$, 2.5 ± 0.36; VR1$^{-/+}$, 4.9 ± 0.40; VR1$^{+/+}$, 2.5 ± 0.23; n = 28). Successful targeting of the locus and germline transmission was confirmed by Southern analysis (Fig. 1b) and by polymerase chain reaction (PCR) (Fig. 1c).

The expression of VR1 in sensory neurons from dorsal root ganglia has been previously established using both messenger RNA analysis and immunocytochemistry[1,2]. In addition, numerous pharmacological assays using vanilloid receptor agonists, such as capsaicin and resiniferatoxin, and antagonists, such as capsazepine[4], have demonstrated the presence of functional VR1 in small diameter dorsal root ganglion (sDRG) neurons[5]. We have used a similar functional approach to confirm the elimination of VR1-mediated responses in our VR1-null mice. Dissociated neuronal cultures were prepared from the DRG of VR1 wild type (VR1$^{+/+}$), VR1 heterozygote (VR1$^{-/+}$) or VR1-null (VR1$^{-/-}$) littermates. After 20–40 h in culture, electrophysiological recordings of responses to a range of stimuli were made from the classically nociceptive, small DRG neurons (diameter <22 μm, mean capacitance 9.4 ± 0.5 pF).

Responses to capsaicin (1 μM) were present in 74% ($n = 34$) of sDRG neurons from $VR1^{+/+}$ mice. A similar number (62%, $n = 21$; data not shown) from $VR1^{+/-}$mice also responded to capsaicin, with currents indistinguishable in size or waveform from those observed in $VR1^{+/+}$ cells. In contrast, capsaicin failed to elicit a response in any of the 29 cells tested from $VR1^{-/-}$cultures (Fig. 2).

In 9 out of 13 cells from $VR1^{+/+}$ cultures a transient extracellular acidification to pH 5.3 produced a slowly developing, non-desensitizing, inward current. This current had kinetics that closely resembled the slow acid-gated current observed in primary rat DRG neurons[6], or in HEK 293 cells or *Xenopus* oocytes expressing VR1 (ref. 1). This slow acid-gated current was not observed in any cells that did not respond to capsaicin. In 46% of cells from $VR1^{+/+}$ cultures, however, an additional rapidly activating and desensitizing inward current was also observed at the onset of a response to pH 5.3. This transient current component was found in both capsaicin-responsive and -unresponsive cells and probably represents the activation of channels belonging to the acid-sensing ionic channel (ASIC) family[7]. In all $VR1^{-/-}$ cells examined, the slowly activating pH 5.3-gated response was absent. In contrast, the rapidly activating ASIC-type current was still seen in a proportion of cells (see Fig. 2a). These data support the hypothesis that a principal part of the maintained (that is, non-desensitizing) acid-gated excitations of sDRG neurons reflects the activity of VR1. This finding also indicates that VR1 may be important in generating acidosis-related pain.

Although lacking the expected responses to pH 5.3 and capsaicin, $VR1^{-/-}$cells responded normally to two neurotransmitters, GABA (γ-aminobutyric acid, 100 μM) and ATP (50 μM), that have been previously shown to activate ion channels in sensory neurons[8,9]. The responses to these agents were present in a similar percentage of $VR1^{+/+}$ and $VR1^{-/-}$ cells (Fig. 2b). Furthermore, no marked differences in response amplitudes or kinetics were noted when recordings from $VR1^{+/+}$ and $VR1^{-/-}$ cells were compared. Notably, the responses to ATP in both $VR1^{+/+}$ and $VR1^{-/-}$ sDRG neurons exhibited the rapid desensitization kinetics typical of capsaicin-responsive sDRG neurons[9].

Heterologously expressed recombinant VR1, in addition to responding to capsaicin, acidic pH and anandamide, is activated by increases in temperature to levels above ~44 °C (refs 1, 10). Similar heat-activated currents have been reported in capsaicin-responsive sDRG neurons[11,12]. To test the contribution of VR1 to these thermal responses, we compared the heat-gated currents generated by 2-s step increases in temperature, from room temperature (23.5–25.5 °C) up to 54 °C, in $VR1^{+/+}$ and $VR1^{-/-}$ sDRG neurons. As reported[11], the heat-activated current exhibits two components (Fig. 3a, top two panels). The first component shares characteristics with currents mediated by recombinant VR1, including a well defined activation threshold at ~44 °C and substantial outward rectification. The second has been ascribed to a 'non-specific' change in membrane or seal resistance[11]. This latter current does not exhibit a marked activation threshold[11], is associated with little or no change in current variance and has an amplitude directly proportional to the pre-stimulus holding current (and input resistance). Such non-specific heat-gated currents can also be observed in capsaicin-unresponsive sDRG neurons as well as in other cell types.

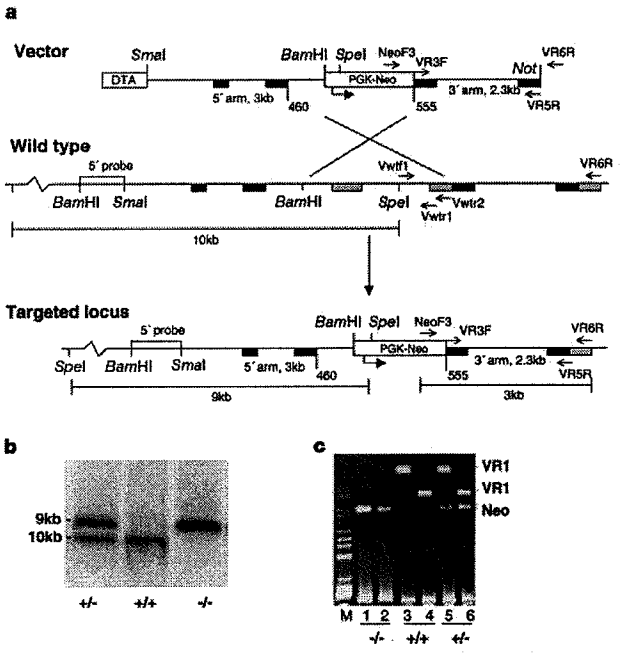

Figure 1 Disruption of VR1 gene by homologous recombination. **a**, Diagram of targeting construct and strategy. Exons are indicated as black boxes. Shaded boxes represent exon sequences not present in the targeting construct. The diagnostic PCR product and *SpeI* cleavage fragments are indicated with the 3′ external PCR primer VR6R and the 5′ external *BamHI*/*SmaI* probe (open box). **b**, Representative example of genomic Southern blot from wild type (+/+), heterozygous mutant (–/+) and homozygous mutant (–/–) mouse tail DNA cleaved with *SpeI* and hybridized with a ^{32}P- labelled *BamHI*/*SpeI* 5′ external probe. **c**, Wild type (+/+), heterozygous mutant (–/+) and homozygous mutant (–/–) mouse tail DNA genotyped by multiplex PCR. All samples contained neo-specific primers. Lanes 1, 3 and 5 contain wild-type VR1-specific primers Vwtf1 and Vwtr1. Lanes 2, 4 and 6 contain wild-type VR1-specific primers Vwtf1 and Vwtr2.

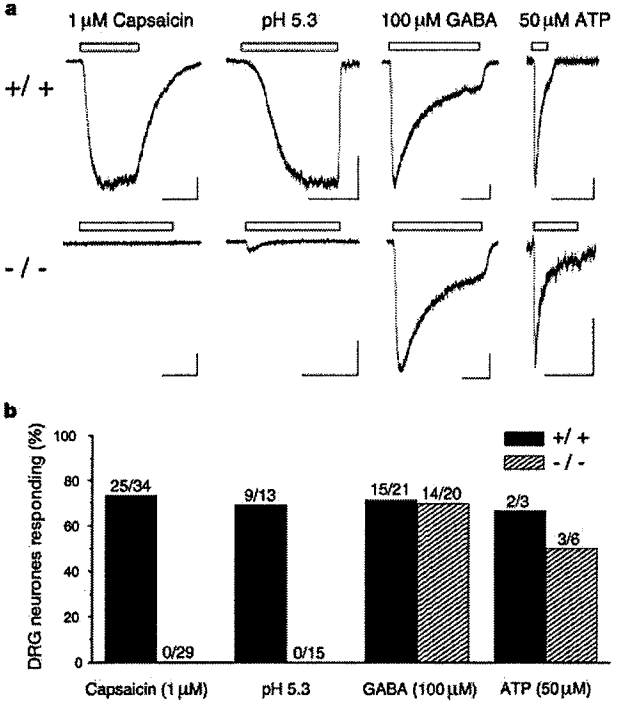

Figure 2 Ligand-gated currents in sDRG neurons from $VR1^{+/+}$ and $VR1^{-/-}$ mice. **a**, Whole-cell patch-clamp recordings of inward currents recorded in sDRG neurons, from $VR1^{+/+}$ or $VR1^{-/-}$ mice, in response to the application of capsaicin (1 μM), protons (extracellular acidification to pH 5.3), GABA (100 μM) or ATP (50 μM) for the time indicated by the bar. Vertical scale bar represents 200 pA for currents recorded in response to capsaicin, pH 5.3 and GABA, and 50 pA for ATP-gated current. The horizontal scale bar represent 5 s in all cases. **b**, Pooled data from all $VR1^{+/+}$(black bars) and $VR1^{-/-}$ (hatched bars) DRG neurons studied indicating the percentage of cells responsive to the application of either capsaicin, GABA or ATP, or yielding a sustained response to protons.

Increases in temperature (from 25 to 54 °C) in both $VR1^{+/+}$ and $VR1^{-/-}$ sDRG neurons consistently produced a heat-activated inward current. In a proportion of $VR1^{+/+}$ cells, the current elicited had an amplitude that was linearly related to the applied temperature (Fig. 3a, top) and was thus attributable to the non-specific current. In the remaining $VR1^{+/+}$ sDRG neurons, a clear additional current component was recruited at temperatures above a threshold of ~44 °C (Fig. 3a, middle, and 3b). The proportion of $VR1^{+/+}$ cells exhibiting this additional heat-gated current (15 out of 33) was similar to the proportion of capsaicin-responsive cells (5 out of 11) in the same preparation (~50%). Pooled data comparing the heat-gated responses at 49–50 °C in $VR1^{+/+}$ with $VR1^{-/-}$ sDRG neurons show that no $VR1^{-/-}$ cells responded with a response greater than 200% of the holding current at room temperature (mean 79 ± 10%), whereas this level was surpassed in almost 50% of $VR1^{+/+}$ cells (Fig. 3c). The lack of a 'specific' current in $VR1^{-/-}$ sDRG neurons strongly supports previous suggestions that a current with its characteristics arises from the thermal activation of VR1 (ref. 12).

On the basis of its heat-dependent gating, VR1 has been proposed to be a sensor for noxious heat[1]. To specifically address this hypothesis, we compared the thermoresponsive behaviour of $VR1^{+/+}$, $VR1^{-/+}$ and $VR1^{-/-}$ mice, both acutely and after the experimental induction of inflammation. The latency of response to an acute thermal stimulus was measured using a 50 or 52.5 °C hotplate (Fig. 4a), or a focused radiant heat stimulus (Fig. 4c,d). All of the mice studied responded to these previously well characterized noxious stimuli.

Increasing the temperature of the hotplate decreased the latency of response for all genotypes (Fig. 4a, $F = 24.91$; d.f. = 1,49; $P < 0.001$). Unexpectedly, neither $VR1^{-/-}$ mice ($P = 0.16$ and $P = 0.06$, 50 and 52.5 °C, respectively) nor $VR1^{+/-}$ mice ($P = 0.52$ and $P = 0.08$, 50 and 52.5 °C, respectively) showed a significant difference when compared with $VR1^{+/+}$mice in hotplate responses (Fig. 4a). Similarly, there were no significant differences between genotypes in terms of response to a radiant heat stimulus set at an intensity that produces a paw-withdrawal latency of ~9 s in normal mice (Fig. 4c). However, the analysis of variance of the effect of genotype upon response latency at 52.5 °C ($F = 3.11$; d.f. = 2,49 $P = 0.053$) only just failed to reach significance. Thus, although it is clear that mice lacking VR1 are still able to respond to noxious heat, there remains a possibility that subtle differences in the behavioural responses to different intensities of heat may occur. Such an effect may be masked to some degree by the mixed strain background of the N1F1 cohort that we used in these studies.

Our data give rise to a paradox because although the electrophysiological response specific to noxious temperatures was absent in the $VR1^{-/-}$ sDRG neurons the null mice responded normally to noxious heat. Neonatal treatment with capsaicin, which ablates the capsaicin-sensitive nociceptors, results in significant analgesia towards thermal stimuli[13], suggesting that the VR1-expressing nociceptors are essential for normal thermal nociception. Future work may help to explain the paradox, for example, by characterization of the DRG cell types responsible for transducing the retained thermal sensation or by the discovery of another heat sensor whose function may be lost or compromised in cell culture. Vanilloid-receptor like 1 (VLR-1), which has also been reported as a heat receptor[14], may contribute to the retained response. VRL-1 has a higher threshold for activation (~52 °C compared with 42 °C for VR1 *in vitro*)[14] and may therefore be unlikely to have a significant

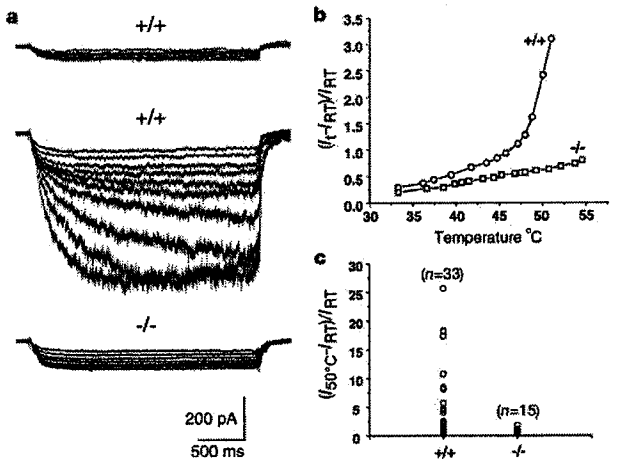

Figure 3 Heat-gated currents in sDRG neurons from $VR1^{+/+}$ and $VR1^{-/-}$ mice. **a**, Example of heat-activated currents from three different sDRGs. Top traces, a $VR1^{+/+}$ cell lacking a detectable threshold-dependent heat-activated current (temperature range 33–54 °C); middle traces, a $VR1^{+/+}$ cell exhibiting a clear threshold-gated current (temperatures 33.3, 37.4, 41.7, 44.8, 45.7, 47.2, 48.8, 50.1, 51 °C); bottom trace, a typical $VR1^{-/-}$ cell (temperature range 33–54 °C). **b**, Plot of applied-heat versus evoked current for the responsive $VR1^{+/+}$ and $VR1^{-/-}$ cells shown in **a**. The peak amplitude for each temperature has been normalized to the pre-stimulus room-temperature (RT) holding current. **c**, Scatter plot showing the increase in current amplitude produced by a temperature jump to 49–50 °C. Data points are normalized to the pre-stimulus room-temperature holding current. Note the significant population of $VR1^{+/+}$ cells exhibiting a heat-gated response greater than 200% of holding current. These cells all possessed a slowly activating high-variance current akin to that shown in the middle panel of **a**.

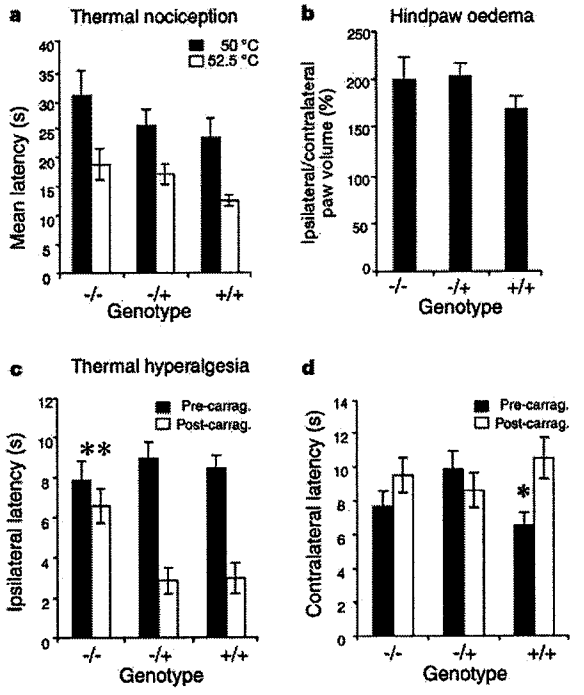

Figure 4 Responses to thermal stimuli in normal and sensitized animals. **a**, Normal nocifensive behaviour to noxious thermal stimuli. Withdrawal or licking latencies in the hotplate assay at 50 or 52.5 °C. No significant differences were seen between wild-type (+/+; $n = 8$ and 9 for 50 °C and 52.5 °C, respectively), heterozygous (–/+; $n = 10$ and 10, respectively), or homozygous mutant (–/–; $n = 10$ and 9, respectively) mice. **b**, Measurement of paw volume after injection of carrageenan. Ipsilateral volume is expressed as a percentage of the uninjected contralateral paw ($n = 10$ for each group). **c**, Ipsilateral withdrawal latencies before or 4 h after carrageenan injection, in response to focused radiant heat. $VR1^{-/-}$ mice showed a highly significant reversal of hyperalgesia when compared with $VR1^{+/+}$ mice; two asterisks, $P < 0.001$. **d**, Contralateral withdrawal latencies before or after carrageenan injection, in response to radiant heat. $VR1^{+/+}$ ($n = 17$) but not $VR1^{-/-}$($n = 18$) or $VR1^{-/+}$($n = 20$) mice show a significant hypoalgesic effect; asterisk, $P < 0.05$.

role in the heat responses described above. Developmental adaptation in the VR1-null mice, or uncharacterized properties of VRL-1 *in vivo*, however, can not be ruled out at this stage. Single or double genetic knockout approaches may shed further light on the relative contributions of VR1, VRL-1 or other molecular heat detectors.

A hyperalgesic response to thermal stimuli is associated with inflammation and was tested for using intraplantar carrageenan injection into the hindpaw and subsequent stimulation by radiant heat[15]. Analysis of the degree of carrageenan-induced hindpaw inflammation showed no significant differences in swelling between $VR1^{-/-}$ and $VR1^{+/+}$ or $VR1^{-/+}$ animals (Fig. 4b). Four hours after carrageenan injection, $VR1^{+/+}$mice and $VR1^{+/-}$ mice exhibited a highly significant decrease in paw-withdrawal latencies compared with baseline pre-inflammation responses (Scheffés test, $P < 0.001$ and $P < 0.001$, respectively). A markedly different result was found in $VR1^{-/-}$ mice. In these animals, withdrawal latencies of inflamed paws were indistinguishable from those measured before carrageenan injection (Scheffés test, $P = 0.955$, Fig. 4c). Thus, knock out of VR1 eliminated the generation of a thermal hyperalgesic response. The contralateral paw showed no significant changes in response latency for the $VR1^{-/-}$ and $VR1^{+/-}$ mice, but there was an increase compared with baseline values in contralateral latencies in $VR1^{+/+}$ mice. This may reflect a chance finding or the onset of an endogenous inhibitory drive resulting in hypoalgesia in the uninflamed contralateral paw (Fig. 4d).

These data highlight a marked effect of VR1 deletion upon responsiveness to noxious thermal stimuli where nociceptive pathways are sensitized (that is, during inflammation), and contrast with the situation in the naive mouse where thermal nociception is relatively unchanged. Pharmacological experiments using the VR1 antagonist capsazepine have also demonstrated an antihyperalgesic effect[16]. Selective VR1 antagonists may therefore prove effective for the treatment of thermal hyperalgesia.

Immunohistochemical and PCR analysis has identified VR1 expression in a wide range of brain regions and has led to suggestions that VR1 might be involved in behaviours other than nociception[3,17]. To screen for further behavioural phenotypes and examine the specificity of our observations, $VR1^{-/-}$ mice were tested for overt behavioural phenotypes using the 'SHIRPA' tests[18]. This assessment included 38 behavioural observations, recording of spontaneous locomotor activity and a holeboard exploratory behaviour test; the data arising from the screen are available in the Supplementary Information. Also included in this screen was a test of mechanical nociception—a toe pinch test. No significant differences between $VR1^{+/+}$ and $VR1^{-/-}$ mice were observed in any of these tests. These negative data demonstrate the specificity of the phenotype resulting from VR1 disruption, and also exclude altered nociceptive responses occurring as a result of effects upon motor function.

Our data demonstrate the specificity of the VR1 knockout in terms of gross behavioural deficits and point to an essential or predominant role of VR1 in responses of sDRG neurons to capsaicin, extracellular acidification or heat. Observations of the activation of VR1 by these and other ligands, such as anandamide[10], suggest that VR1 may also function in pain where these other mediators are the predominant agonists. Our experiments have focused upon thermal responses, however, and the results show conclusively that VR1 is essential for the development of sensitization to thermal stimuli during inflammation but not for the normal sensation of noxious heat. □

Methods

Targeting of VR1 gene and generation of mutant mice

A standard gene targeting approach was chosen to replace, in E14.1 ES cells, the DNA encoding amino acids 460–555 of mVR1 with the neomycin phosphotransferase gene (detailed methods are provided in Supplementary Information). A 2.3-kilobase (kb) 3′ and 3.75-kb 5′ homology arm were isolated from a mouse 129SVJ BAC library, cloned either side of PGKneo, and flanked by Diptheria toxin-A gene as a negative selection marker[19]. Homologous recombination in resistant ES cells was confirmed by Southern blot and a multiplex PCR genotyping procedure[20,21]. Three targeted clones were injected into C57Bl6/J-derived blastocysts, and the resulting chimaeras produced germline offspring. Males heterozygous for the mutated allele were mated to C57Bl6/J females and mutant progeny were intercrossed to generate an N1F1 study population. All experiments were conducted according to the requirements of the United Kingdom Animals (Scientific Procedures) Act (1986) and conformed to the ethical standards of SmithKline Beecham Pharmaceuticals.

Electrophysiological recordings

Dissociated DRG cells were prepared from 8–10-day-old pups and cultured on glass coverslips[5] in DMEM with N2 supplements, 50 ng ml^{-1} β-NGF, 0.05% bovine serum albumin, 100 U ml^{-1} penicillin and streptomycin. Whole-cell patch-clamp recordings were made from sDRGs (≤22 μM diameter) with an Axopatch 200B amplifier controlled by the pClamp7 software suite (Axon Instruments). For analysis of ligand- and pH-gated current, all experiments were conducted at room temperature (23–25.5 °C) at a holding potential of −70 mV. The bath solution comprised (in mM) NaCl 130, KCl 5, $CaCl_2$ 2, $MgCl_2$ 1, glucose 30, HEPES-NaOH 25; pH 7.3. Corresponding pH 5.3 solutions were adjusted with HCl. To minimize the holding current, and thus the contribution of the 'non-specific' heat-gated currents, most of our recordings of heat-gated current were made in near symmetrical Cs^+concentrations, with extracellular Ba^{2+} substituted for Ca^{2+} and methanesulphonate as the predominant intracellular anion. However, essentially similar observations (data not shown) were also made in a population of recordings under the conditions above. Electrodes had a resistance of 2–7 MΩ when filled with (in mM): CsCl 140, $MgCl_2$ 4, EGTA 10, HEPES-CsOH 10; pH 7.3. Applications of agonists and room temperature to 54 °C temperature jumps were made using an automated fast-switching solution exchange system (Warner Instruments SF-77B; time for solution exchange ~30 ms), in a manner similar to that described elsewhere[11].

Behavioural studies

All tests were performed on male 6–9-week-old mice (n = 10). $VR1^{-/-}$ and $VR1^{+/+}$ littermates were used in the primary behavioural observation screen SHIRPA[18], full experimental procedures for which are available at http://www.mgu.har.mrc.ac.uk/mutabase/. In addition, a 30-min spontaneous locomotor activity test and a 10-min holeboard test of exploratory activity were used[22].

Hotplate test

Mice of each genotype (n = 8–10) were tested in a random and blind fashion for thermal nociception using a hotplate (Harvard Analgesia Meter, Harvard Instruments) maintained at 50 or 52.5 °C. Mice were observed for signs of nociception, that is, rapid fanning or licking of the paws. The response latency was recorded and results analysed using 2-way analysis in Statistica (Statsoft Inc.). Genotype and hotplate temperature were used as independent variables. Follow up analyses were carried out using Duncan's test.

Carrageenan-induced inflammation

The same cohort of mice (n = 17–20 per genotype) were tested for thermal hyperalgesia using described methods modified for mice[14]. Animals were habituated to the test apparatus. Baseline withdrawal latencies, to a focused radiant heat stimulus (Plantar Test Apparatus, Ugo Basile), were measured for each hindfoot. Carrageenan (0.025 mls, 2%) was injected sub-plantar into the left hindfoot, and paw-withdrawal latencies were measured for both hindfeet 4 h later. Paw volumes were measured using a plethysmometer (Linton Instruments). Results were expressed as mean latency (s) for paw withdrawal at baseline and after carrageenan injection. All data were log transformed to correct for heterogeneity of variances. Results were analysed in SAS (Statsoft Inc.) using a split plot analysis of variance. Genotype and measurement were used as independent variables. In both cases, follow up analysis was carried out using Scheffés test, where appropriate[23]. Errors shown represent standard errors of the mean.

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Supplementary information is available on *Nature*'s World-Wide Web site (http://www.nature.com) or as paper copy from the London editorial office of *Nature*.

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Glutamatergic synapses on oligodendrocyte precursor cells in the hippocampus

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Fast excitatory neurotransmission in the central nervous system occurs at specialized synaptic junctions between neurons, where a high concentration of glutamate directly activates receptor channels. Low-affinity AMPA (α-amino-3-hydroxy-5-methyl isoxazole propionic acid) and kainate glutamate receptors are also expressed by some glial cells[1], including oligodendrocyte precursor cells (OPCs). However, the conditions that result in activation of glutamate receptors on these non-neuronal cells are not known. Here we report that stimulation of excitatory axons in the hippocampus elicits inward currents in OPCs that are mediated by AMPA receptors. The quantal nature of these responses and their rapid kinetics indicate that they are produced by the exocytosis of vesicles filled with glutamate directly opposite these receptors. Some of these AMPA receptors are permeable to calcium ions, providing a link between axonal activity and internal calcium levels in OPCs. Electron microscopic analysis revealed that vesicle-filled axon terminals make synaptic junctions with the processes of OPCs in both the young and adult hippocampus. These results demonstrate the existence of a rapid signalling pathway from pyramidal neurons to OPCs in the mammalian hippocampus that is mediated by excitatory, glutamatergic synapses.

Oligodendrocytes in the mammalian central nervous system develop from a population of precursor cells during late gestational and early postnatal life[2], providing the insulating sheaths of myelin necessary for rapid conduction of action potentials along axons. These precursors or OPCs were identified anatomically as smooth protoplasmic astrocytes based on their unique stellate morphology[3], and their properties have been studied both in culture (termed O-2A cells)[4–6] and in acutely isolated tissue (termed glial precursors or complex cells)[1,7]. Glutamate receptor activation in these cells inhibits their proliferation and maturation into oligodendrocytes[8], and prolonged exposure to glutamate causes excitotoxic degeneration[9]. Despite the potential importance of this pathway in the development and regeneration of myelin, it is not known how these receptors are activated *in vivo*. Glutamate has been shown to reach other glial cells by diffusion from nearby synaptic clefts following vesicular release[10], or by reverse transport along axons[11].

To determine how glutamate reaches AMPA receptors on OPCs, we made whole-cell patch-clamp recordings from OPCs in the hippocampus and measured their response to stimulation of afferent excitatory axons. OPCs located in the stratum radiatum region of area CA1 exhibited small Na^+ currents, large A-type and delayed rectifier K^+ currents, and did not fire action potentials (Fig. 1a) (n = 28). Electrical stimulation in stratum radiatum elicited inward currents in OPCs that had rapid kinetics (Fig. 1b). Cells with these properties had a stellate morphology, with thin, highly branched processes that extended from a small cell body (Fig. 1c). They were identified as OPCs by their immunoreactivity to NG2 (Fig. 1d, e, n = 10/10), a proteoglycan that is only expressed by OPCs in this region[12]. These NG2-positive cells were immunonegative for glial fibrillary acidic protein (n = 3/3), while astrocytes recorded from under similar conditions were immunopositive (n = 8/8 groups of cells).

Paired stimuli produced currents in OPCs that were larger (P2/P1 = 1.7 ± 0.1, n = 16) and exhibited fewer apparent failures following the second stimulus (Fig. 2a), similar to paired-pulse facilitation of excitatory postsynaptic currents (EPSCs) in CA1

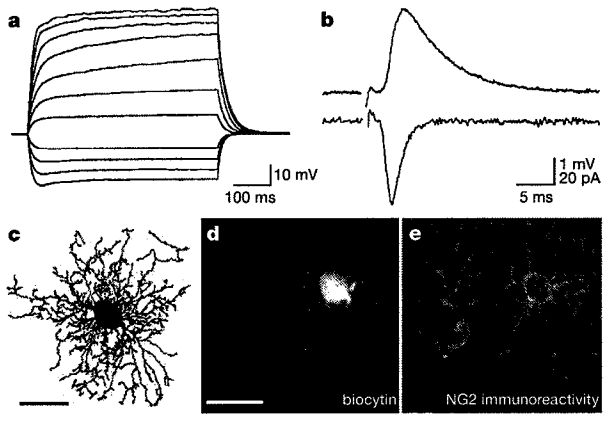

Figure 1 Synaptic responses from identified OPCs in hippocampal slices. **a**, Current-clamp recording of membrane responses to current injection (–80 to 140 pA; step size, 20 pA). **b**, Evoked responses to Schaffer collateral/commissural fibre stimulation, recorded in voltage-clamp (holding potential, –90 mV; lower trace) and current-clamp (membrane potential = –90 mV; upper trace). Traces are averages of 15 consecutive responses recorded from the same cell. Stimulus: 30 μA, 100 μs. **c**, Reconstruction of a biocytin-filled OPC. **d**, Micrograph of the same OPC as in **d**, visualized by AMCA-conjugated streptavidin. **e**, NG2-immunoreactivity of same region of the slice as shown in **d**. Scale bars for **c** and **d**, 20 μm; **d** and **e** are at the same magnification.

EXHIBIT B

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The Cloned Capsaicin Receptor Integrates Multiple Pain-Producing Stimuli

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Summary

Capsaicin, the main pungent ingredient in "hot" chili peppers, elicits burning pain by activating specific (vanilloid) receptors on sensory nerve endings. The cloned vanilloid receptor (VR1) is a cation channel that is also activated by noxious heat. Here, analysis of heat-evoked single channel currents in excised membrane patches suggests that heat gates VR1 directly. We also show that protons decrease the temperature threshold for VR1 activation such that even moderately acidic conditions (pH ≤ 5.9) activate VR1 at room temperature. VR1 can therefore be viewed as a molecular integrator of chemical and physical stimuli that elicit pain. Immunocytochemical analysis indicates that the receptor is located in a neurochemically heterogeneous population of small diameter primary afferent fibers. A role for VR1 in injury-induced hypersensitivity at the level of the sensory neuron is presented.

Introduction

In mammals, the initial detection of noxious chemical, mechanical, or thermal stimuli, a process referred to as nociception, occurs predominantly at the peripheral terminals of specialized, small diameter primary afferent neurons, called polymodal nociceptors (Fields, 1987). These afferents transmit the information into the CNS, ultimately evoking a perception of pain or discomfort and initiating appropriate protective reflexes. The protective function of this system is predicated on its ability to detect diverse physical and chemical stimuli, distinguish between noxious and innocuous events by setting specific response thresholds for a given stimulus, and reset these thresholds to sensitize the system and guard against further injury. A fundamental goal in pain biology is to understand how disparate physical and chemical stimuli are qualitatively and quantitatively sensed at the nociceptor terminal under normal and pathophysiological conditions. Do noxious thermal, mechanical, and chemical stimuli excite nociceptors through shared or distinct signal transduction pathways? How does a response to one type of stimulus alter sensitivity to others? To address these questions, it is necessary to identify the molecular entities at the nociceptor terminal that detect noxious signals and transduce this information into membrane depolarization events.

Our approach to identifying such molecules has been to elucidate the mechanism whereby plant-derived agents, such as capsaicin, resiniferatoxin, and other vanilloid-containing compounds, elicit a sensation of burning pain. Vanilloid compounds selectively depolarize nociceptors (Bevan and Szolcsanyi, 1990; Szolcsanyi, 1993; Szallasi, 1994), presumably by mimicking the actions of a physiological stimulus or endogenous ligand that activates the "nociceptive" pathway. We have recently determined the molecular basis underlying this phenomenon by characterizing a functional cDNA that encodes a vanilloid receptor (VR1) in rat sensory ganglia (Caterina et al., 1997). VR1 is a vanilloid-gated, nonselective cation channel that resembles members of the transient receptor potential (TRP) channel family, first identified as components of the *Drosophila* phototransduction pathway (Montell and Rubin, 1989). The restriction of VR1 expression to small diameter neurons within sensory ganglia is sufficient to account for the highly selective nature of vanilloid compounds as excitatory agents for nociceptors. Remarkably, *Xenopus* oocytes or transfected mammalian cells expressing VR1 exhibit robust membrane currents not only in response to capsaicin but also to increases in ambient temperature capable of producing pain in humans (i.e., noxious heat). Because vanilloid- and heat-evoked currents are cation selective, have similar outwardly rectifying current–voltage relations, and are blocked by the noncompetitive capsaicin receptor antagonist ruthenium red, we have postulated that VR1 mediates responses to both stimuli in vitro and in vivo (Caterina et al., 1997). However, the relationship between heat- and vanilloid-evoked currents at the whole-cell and single channel levels remains unclear. Moreover, it has yet to be established whether heat activates VR1 directly or through other thermally sensitive molecules. Finally, a number of studies conducted in cultured sensory neurons have provided evidence for heterogeneity among heat-evoked currents (Cesare and McNaughton, 1996; Reichling and Levine, 1997), and the relationship of VR1 to these activities remains to be resolved.

In addition to heat and vanilloid compounds, protons also influence vanilloid receptors and nociceptive pathways. High proton concentrations (pH < 6) are generated during various forms of tissue injury, including infection, inflammation, and ischemia (Jacobus et al., 1977; Stevens et al., 1991; Steen et al., 1992; Bevan and Geppetti, 1994). Such acidification can elicit pain or sensitize the affected area (Steen et al., 1992; Steen and Reeh, 1993; Bevan and Geppetti, 1994). In the latter case, a stimulus above the normal pain threshold can evoke an exaggerated pain response, a condition referred to as hyperalgesia (Fields, 1987; Dubner and Basbaum, 1994; Meyer et al., 1994). Protons are likely to produce or exacerbate pain via their interaction with several receptors and channels on nociceptive sensory neurons, including acid-sensitive ion channels of the

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degenerin family (Waldmann et al., 1997a, 1997b), ATP-gated ion channels (Stoop et al., 1997), and vanilloid receptors (Petersen and LaMotte, 1993; Kress et al., 1996; Caterina et al., 1997). In cultured sensory neurons, protons potentiate capsaicin-evoked responses (Petersen and LaMotte, 1993; Kress et al., 1996). At higher concentrations, protons by themselves elicit membrane currents (Bevan and Yeats, 1991; Kress et al., 1996), a component of which has been postulated to result from their direct or indirect activation of vanilloid receptors (Bevan and Yeats, 1991; Bevan and Geppetti, 1994).

With the cloned capsaicin receptor in hand, we can now directly address the mechanisms of action of heat, protons, and vanilloid compounds on the receptor and explore the relationships among these stimuli at the cellular and molecular levels. In the present study, we provide evidence, in the form of single channel recordings from excised membrane patches and simultaneous current and temperature recordings in oocytes, that heat activates VR1 directly and dynamically without the involvement of cytoplasmic components. We also show that protons dramatically potentiate the effects of heat and capsaicin on VR1 activity by decreasing the threshold for channel activation by either agonist. In fact, at physiologically attainable proton concentrations (pH < 5.9), the channel opens at room temperature. In light of these observations, we present a model wherein the vanilloid receptor functions as a polymodal signal detector whose activity reflects the combined status of multiple physiological stimuli. Finally, using antisera directed against the cloned receptor, we provide evidence that VR1 is expressed both centrally and peripherally within somatic and visceral unmyelinated primary afferent nociceptors. We also show that VR1 is not uniformly expressed in peptide- (substance P) and non-peptide- (lectin IB4) containing dorsal root ganglion (DRG) neurons, which suggests that there is a heretofore undetected physiological heterogeneity among polymodal nociceptors. The anatomical distribution and functional properties that we describe are consistent with VR1 contributing to both thermosensation and chemosensation in vivo. Moreover, the ability of VR1 to detect and integrate information from physical and chemical inputs is a property expected of a signal transduction molecule that assesses the physiological environment of the sensory nerve terminal and alters neuronal responsiveness in the context of tissue injury.

Results

Functional Comparison of Capsaicin- and Heat-Activated Responses

We conducted a series of electrophysiological and pharmacological analyses to better understand the functional relationship between capsaicin- and heat-evoked VR1 currents. In transfected human embryonic kidney–derived HEK 293 cells expressing VR1, we found that the magnitudes of capsaicin-evoked (1 μM) and heat-evoked (46°C) currents in individual cells were significantly correlated ($r = 0.82$, $p < 0.0001$, least squares regression, $n = 26$; data not shown), with heat-evoked responses being ~25% the size of those evoked by capsaicin. This correlation is in good agreement with the reported concordance between heat- and capsaicin-evoked responses in cultured sensory neurons (Kirschstein et al., 1997) and is consistent with VR1 being a direct effector for both stimuli. Second, we asked whether activation by one agonist would cross-desensitize VR1 to the other agonist. To answer this question, we initially examined responses in the absence of extracellular calcium, where repetitive capsaicin-evoked currents do not desensitize (Caterina et al., 1997) but repetitive heat-evoked currents show significant desensitization (Figure 1A). Under these conditions, we found that heat produced profound cross-desensitization to subsequent challenges with capsaicin (Figure 1A). Similarly, we found that, in the presence of extracellular calcium, capsaicin treatment desensitized VR1-expressing cells not only to subsequent challenges with capsaicin but also to heat (Figure 1A). Interestingly, desensitization to either agonist could be overcome by applying both stimuli simultaneously. The resulting responses desensitized in a calcium-dependent manner, similar to the behavior of capsaicin responses in naive cells. Third, we asked whether heat-evoked currents exhibited the distinctive cation permeability sequence ($Ca^{2+} > Mg^{2+} > Na^{+}$) that is characteristic of capsaicin-evoked currents (Bevan and Szolcsanyi, 1990; Caterina et al., 1997). Ion substitution experiments revealed that heat-evoked currents show a similar preference for divalent cations, albeit with somewhat different relative permeabilities: for heat, the ratios were $P_{Ca}/P_{Na} = 3.8 \pm 0.3$, $n = 6$, and $P_{Mg}/P_{Na} = 3.1 \pm 0.4$, $n = 6$ (data not shown); for capsaicin, they were $P_{Ca}/P_{Na} = 9.6$ and $P_{Mg}/P_{Na} = 5.0$ (Caterina et al., 1997). Fourth, we tested whether antagonists that block capsaicin-evoked responses would also block heat-evoked currents. Consistent with our previous observations in oocytes, we found that both the noncompetitive antagonist ruthenium red (10 μM) and the competitive antagonist capsazepine (10 μM) significantly blocked capsaicin-evoked responses in VR1-expressing HEK 293 cells (Figure 1B). Likewise, heat-activated currents in transfected cells were virtually abolished in the presence of these antagonists, compared with a 44% decrease (attributable to desensitization between the first and second heat challenges) in the absence of antagonist (Figures 1B and 1C). Moreover, capsazepine (10 μM) produced a significant blockade of heat-activated currents in VR1-expressing *Xenopus* oocytes that partially recovered following antagonist washout (Figure 1C). Taken together, these observations strongly support the conclusion that VR1-mediated responses to capsaicin and heat involve distinct but overlapping mechanisms.

Heat Activates VR1 by a Membrane Delimited and Graded Mechanism

If VR1 is, itself, a heat sensor, then heat-evoked responses should occur in isolated membrane patches, free of soluble cytoplasmic components. Indeed, well-resolved heat- or capsaicin-evoked currents were observed in patches excised from VR1-transfected (but not vector-transfected) HEK 293 cells in the inside-out (Figures 2A and 2B) or outside-out (data not shown)

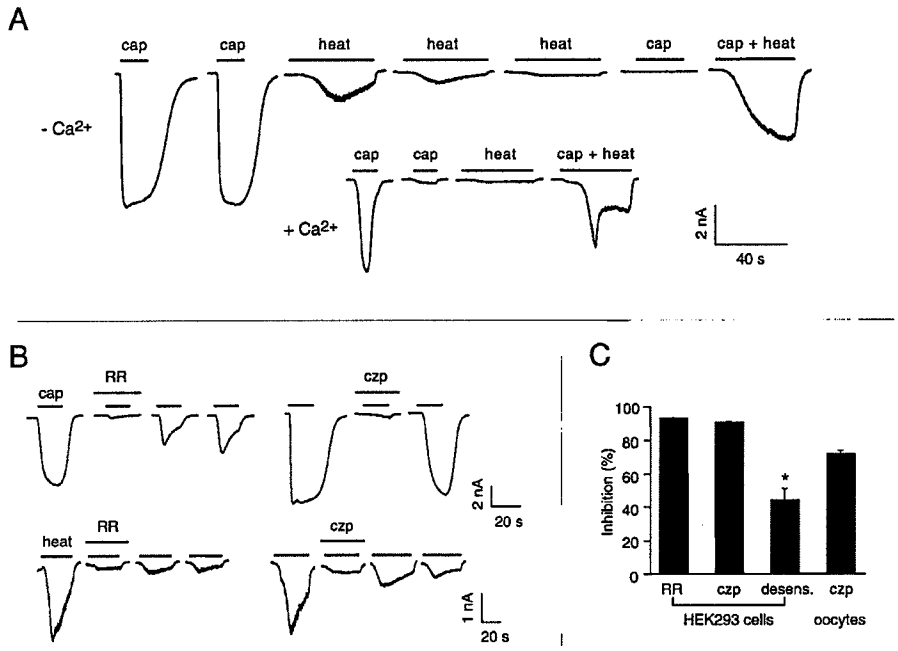

Figure 1. The Cloned Capsaicin Receptor Is an Effector for Both Vanilloid Compounds and Heat

(A) Capsaicin and heat show cross-desensitization and synergistic recovery. Whole-cell current responses were evoked by capsaicin (cap) or heat in VR1-transfected mammalian HEK 293 cells bathed in Ca^{2+}-free or Ca^{2+}-containing solutions. Desensitization produced by 1 μM capsaicin in Ca^{2+}-containing solution could not be overcome by application of 100 μM capsaicin alone (data not shown). No significant heat-evoked currents were observed in cells expressing $5HT_3$ (n = 5) or $P2X_2$ (n = 5) ligand-gated channels (data not shown).

(B and C) Vanilloid receptor antagonists block both capsaicin- and heat-evoked currents. Antagonism by capsazepine (czp, 10 μM) or ruthenium red (RR, 10 μM) was assessed following a 30 s antagonist pretreatment period for capsaicin-evoked responses and a 2 min pretreatment period for heat-evoked response. Capsaicin-activated currents in HEK 293 cells were blocked by RR (98.1% ± 1.0%, n = 7) or czp (97.3% ± 1.7%, n = 7). Heat-activated currents in HEK 293 cells were also inhibited by RR (92.2% ± 1.0%, n = 4) and czp (90.3% ± 0.5%, n = 5). Decrease of heat-activated currents in the absence of antagonist due to receptor desensitization between the first and second heat challenges (desens.) in HEK 293 cells (43.7% ± 7.3%, n = 8) was significantly smaller than the apparent inhibition in the presence of antagonist (*$p < 0.001$, unpaired t test). Heat-activated currents in oocytes were inhibited by czp (71.2% ± 2.9%, n = 11). Capsaicin concentration was 1 μM and bath temperature was elevated from ~22°C to 46°C in 25 s (monitored using an in-bath thermocouple) and from ~22°C to 44°C in HEK 293 cells and oocytes, respectively. Holding potentials (E_h) were −60 mV and −40 mV in HEK 293 cells and oocytes, respectively. The interstimulus interval was 2 min; bars indicate the duration of agonist stimuli.

configuration. These single channel responses displayed a number of properties in common: (1) both showed reversible inhibition by capsazepine (10 μM), as reflected in a significant reduction in the mean channel activity (NP_o) (Figure 2A); (2) both exhibited outwardly rectifying current–voltage relations (Figure 2B and Caterina et al., 1997); and (3) single channel Na^+ conductances derived from these current–voltage relations were similar for heat- and capsaicin-evoked currents (83 and 77 pS, respectively, at positive potentials; Figure 2B and Caterina et al., 1997). These rectification and conductance properties resemble those determined for native heat-activated currents in cultured sensory neurons (Cesare and McNaughton, 1996; Reichling and Levine, 1997; H. Rang, personal communication).

If VR1 directly senses heat stimuli, then one might also expect heat-activated currents to vary directly as a function of ambient temperature. In oocytes expressing VR1, heat-evoked currents were remarkably responsive to changes in bath temperature above 40°C. Continuous increases and decreases in temperature produced coordinate changes in current, as if the channel were acting as a molecular thermometer (Figure 2C). Taken together with the single channel data, these findings indicate that any mechanism underlying VR1 activation must involve a membrane delimited signaling event in which channel responses are temporally linked to changes in thermal stimuli. In the simplest scheme, VR1 is an intrinsically heat-sensitive channel that can respond to thermal stimuli in any cellular environment.

Protons Potentiate Both Capsaicin- and Heat-Evoked Responses

The observation that a drop in bath pH (pH_o) augments responses of cultured sensory neurons to capsaicin (Petersen and LaMotte, 1993; Kress et al., 1996) has raised many questions about the pharmacological mechanism that underlie this phenomenon. Is it relevant, for instance, to the process whereby acidosis exacerbates pain? In initial experiments, we found that protons enhance the response of VR1-expressing oocytes to capsaicin (Caterina et al., 1997). The same effect is seen in VR1-transfected mammalian cells, and dose–response

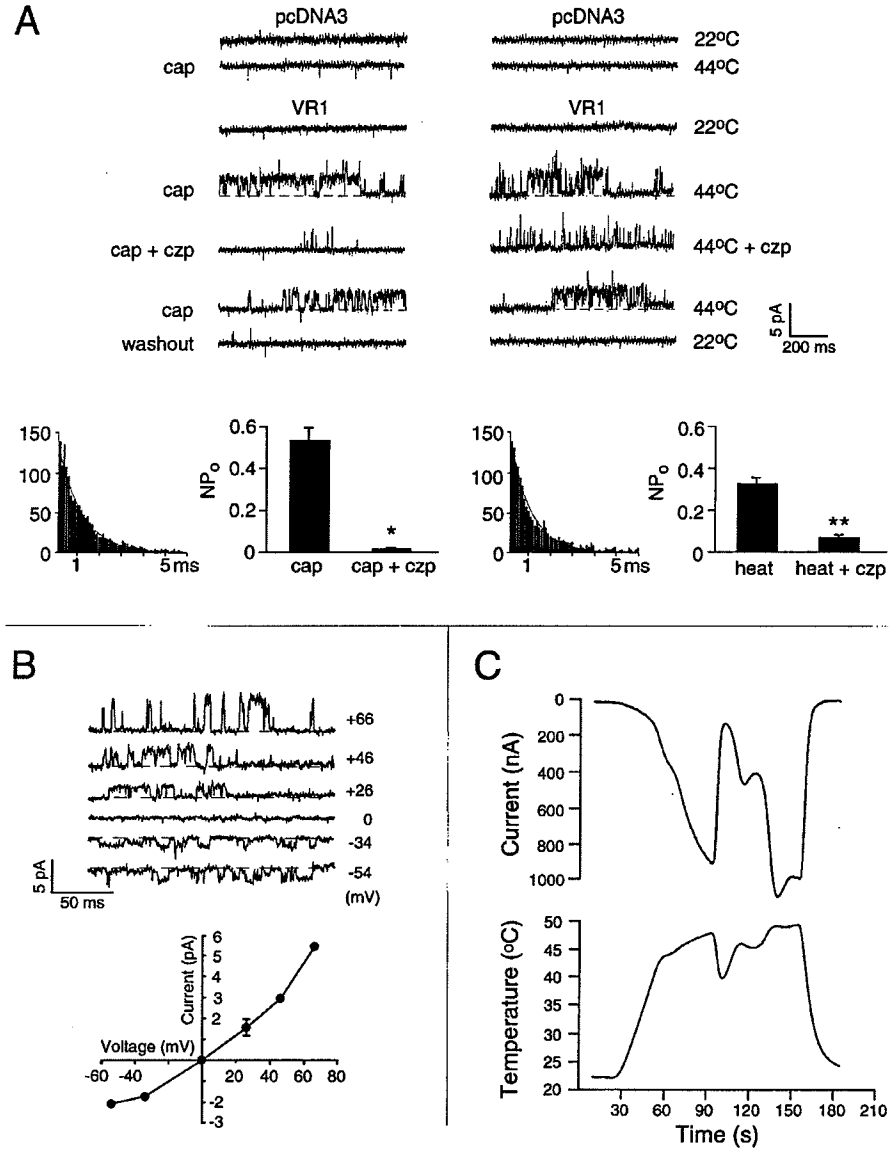

Figure 2. Heat Activates VR1 through a Membrane-Delimited and Graded Mechanism

(A) Capsaicin (cap, left, 100 nM) and heat (right, 44°C) evoke single channel openings in VR1-transfected but not in vector-transfected (pcDNA3) control cells. Single channel activity elicited by capsaicin was significantly blocked by capsazepine (czp, 10 μM, NP_o reduced by 97.7% from 0.528 ± 0.058 to 0.012 ± 0.004, n = 7, *$p < 0.0001$, paired t test) as was heat-evoked activity (NP_o reduced by 79.8% from 0.321 ± 0.029 to 0.065 ± 0.012, n = 11, **$p < 0.00001$, paired t test). Unitary amplitudes were 2.87 ± 0.09 pA for capsaicin (+40 mV, n = 10) and 2.95 ± 0.07 pA for heat (+46 mV, n = 28). Open time histograms from representative patches are shown at bottom; time bins are 0.1 ms. Time constants obtained by fitting histograms to single exponentials were not significantly different for capsaicin (0.98 ± 0.10 ms, n = 4) and heat (0.86 ± 0.04 ms, n = 5, unpaired t test). For heat-activated currents, membrane potentials were corrected for the junctional potential change measured at 44°C (6.3 ± 0.4 mV, n = 6). Broken lines indicate closed channel level.

(B) Heat-evoked single channel currents show outward rectification. Representative single channel traces of heat-activated currents (44°C) at indicated membrane potentials are shown (top) together with derived current–voltage curve of mean single channel amplitudes (± SEM) (bottom, n = 5–28 patches). Broken lines indicate closed channel level. Single channel chord conductance for Na^+ between 0 and +66 mV was 83.4 ± 2.9 pS (n = 5).

(C) Fluctuations in bath temperature above 40°C elicit dynamic and synchronized current changes in voltage-clamped VR1-expressing oocytes.

curves generated under neutral (pH 7.4) and acidic (pH 6.4) bath conditions clearly demonstrated that protons potentiate capsaicin action by increasing its potency (EC_{50} = 111 nM and 60 nM, respectively) without altering efficacy (Figure 3A). Under these moderately acidic conditions, protons serve only as modulatory agents because no current was observed in the absence of capsaicin (data not shown).

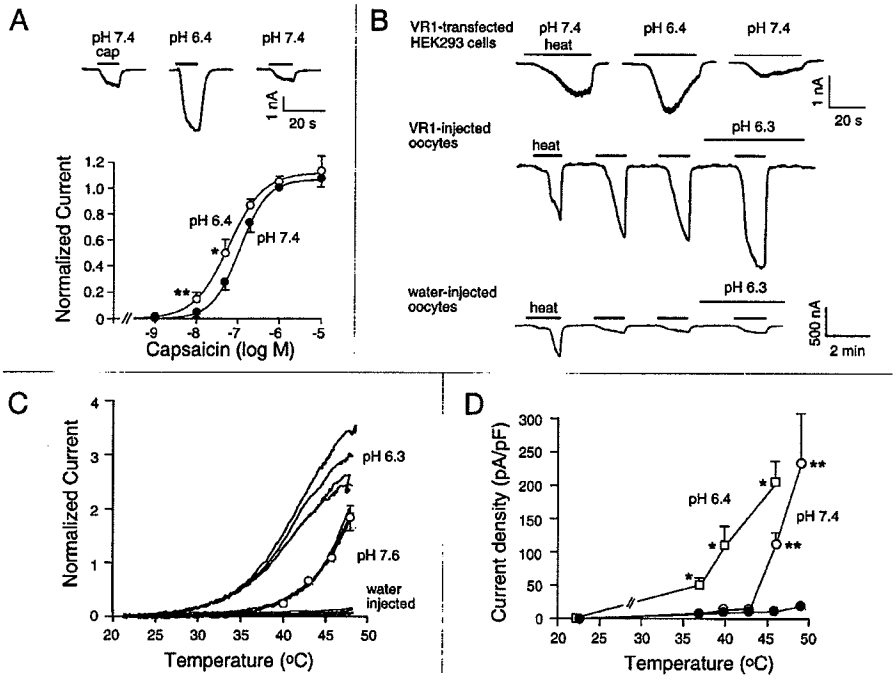

Figure 3. Protons Potentiate Capsaicin- and Heat-Activated VR1 Currents

(A) A change in extracellular pH from 7.4 to 6.4 reversibly potentiates whole-cell currents activated by capsaicin (cap, 50 nM) at room temperature. Capsaicin (bars) was applied at 2 min intervals, and cells were pretreated with pH 6.4 bath solution for 2 min. Concentration–response curves for capsaicin in bath solutions of pH 7.4 (closed circles) and pH 6.4 (open circles) exhibit a 1.9-fold difference in agonist potency. EC_{50} values were 111 nM (SD = 16 nM) and 60 nM (SD = 14 nM); Hill coefficients were 1.32 (SD = 0.21) and 1.02 (SD = 0.21), respectively. Normalized whole-cell currents (mean ± SEM, n = 14) are expressed as a fraction of the response evoked in each cell by 1 μM capsaicin at pH 7.4. Asterisk and double asterisk indicate significant differences between pH 7.4 and pH 6.4 (*$p < 0.05$ and **$p < 0.01$, unpaired t test).

(B) Protons potentiate heat-activated currents in VR1-expressing HEK 293 cells (top) and oocytes (middle) held at −60 and −40 mV, respectively. In HEK 293 cells, bath temperature was elevated to 46°C as in Figure 1. Pretreatment time with pH 6.4 bath solution was 2 min. In oocytes, bath temperature was elevated from ~22°C to 48°C over 90 s. Heat-evoked responses in oocytes, which increased in size between the first and second stimuli, stabilized by the third stimulus. Water-injected control oocytes (bottom) sometimes exhibited an initial heat-evoked current that was markedly diminished during subsequent challenges. Room temperature bath solution at pH 6.3 evoked no response in VR1- or water-injected oocytes (data not shown).

(C and D) Protons lower the threshold and increase the magnitude of heat-evoked currents in VR1-expressing cells. Temperature- response curves in oocytes (C) were derived from the increasing temperature phase of the third (pH 7.6) and fourth (pH 6.3) heat-evoked responses in cells treated as in (B). For a given VR1-expressing oocyte (n = 4), currents were normalized to responses at 45°C (pH 7.6). For water-injected control oocytes (n = 4), the third (pH 7.6) heat-evoked response was plotted as a fraction of the average 45°C, pH 7.6 stimulus-evoked current among VR1-expressing oocytes. Open circles indicate mean (± SEM) normalized current measured after 30 s of continuous exposure to fixed temperatures (n = 4). Temperature-response curves in transfected HEK 293 cells (D) were derived in bath solutions of fixed temperatures at pH 7.4 (open circles, n = 6–17) or pH 6.4 (open squares, n = 7–9). Vector-transfected cells were similarly treated at pH 7.4 (closed circles, n = 5–7). Heat (46°C) evoked no responses at low pH in vector-transfected HEK 293 cells (data not shown). Each point represents mean values (± SEM). Current was normalized to membrane capacitance. Asterisks and double asterisks indicate significant difference from VR1-expressing cells at pH 7.4 and from vector-transfected cells at pH 7.4, respectively ($p < 0.01$, unpaired t test).

Of greater physiological relevance, of course, is whether protons modulate the response of VR1 to heat. In fact, we found that a decrease in extracellular pH significantly potentiates heat-evoked responses in either VR1-expressing HEK 293 cells or *Xenopus* oocytes (Figure 3B). To determine whether this effect occurs over a range encompassing both noxious and nonnoxious temperatures, we generated continuous temperature-response curves from oocytes expressing VR1. In this system, membrane currents were recorded from voltage-clamped oocytes while the temperature of the perfusate solution was elevated from ~22°C to 48°C. Unlike mammalian cells, oocytes expressing VR1 exhibited minimal desensitization of heat-evoked responses. As a result, a thermal response curve generated during a continuous temperature ramp was superimposable with one defined by currents obtained at several steady state temperatures (Figure 3C). In other words, continuous temperature ramps can be used to generate accurate thermal response profiles of VR1 in oocytes. These temperature-response curves illustrate two interesting and significant points. First, a reduction in pH_o produces markedly larger responses at temperatures that are noxious to mammals. Second, a reduction in pH_o dramatically lowers the threshold for channel activation, such that at pH_o 6.3, substantial currents can be seen at temperatures as low as 35°C (where the channel is normally closed at pH_o 7.6). We observed a similar phenomenon

in VR1-transfected mammalian cells exposed to heat stimuli at fixed temperatures (Figure 3D). Here, a clear leftward shift of the temperature–response curve was also seen with a reduction in bath pH. Notably, the threshold for heat-evoked responses at pH_o 7.4 was ~43°C, in excellent agreement with reported thermal response thresholds for heat-activated currents in cultured rat sensory neurons (Cesare and McNaughton, 1996), subjective pain ratings in humans, and pain-associated single-fiber activity in monkeys (LaMotte and Campbell, 1978; Meyer and Campbell, 1981; Meyer et al., 1994). Moreover, the graded response of VR1 above this threshold is analogous to progressively larger physiological responses observed in these systems with further increases in temperature.

Protons Activate VR1 at Normal Physiological Temperatures

To more precisely mimic a physiological scenario in which inflammation or ischemia lowers tissue pH at body temperature, we exposed VR1-transfected cells to an abrupt decrease in pH_o (to 6.4) at a constant temperature of 37°C, which is well below the temperature threshold for activating VR1 at normal physiological pH. Under these circumstances, a distinct proton-evoked current was observed (Figure 4A), demonstrating that at the nonelevated body temperature of 37°C, protons can act as VR1 agonists. In light of this observation, we examined the effects of protons over a broader range, speculating that at sufficiently high concentrations, hydrogen ions might further shift the VR1 thermal response profile, rendering the channel open at room temperature. As predicted, sustained proton-evoked responses were reproducibly observed at pH_o 5.4 in VR1-transfected (but not vector-transfected) mammalian cells at 22°C. Responses to capsaicin and protons shared several characteristics, including calcium-dependent desensitization profiles, reversible inhibition by capsazepine, and outwardly rectifying current–voltage relations (Figure 4B and Caterina et al., 1997). A dose–response curve generated at 22°C exhibited a threshold pH of ≤5.9 with a half-maximal effective pH of 5.4 (Figure 4C). We estimate that saturating proton concentrations produce maximal responses that are 20%–30% of the magnitude of responses obtained with a saturating dose of capsaicin (1.0 μM), which suggests that protons behave as partial agonists of VR1 with respect to capsaicin. We previously reported that <10% of VR1-expressing oocytes showed currents upon exposure to pH 5.5 bath solution (Caterina et al., 1997). Here, we found that when pH_o was reduced to 4.0, responses were uniformly observed (data not shown). The reason for the reduced sensitivity of VR1 to protons in this system is not clear but could reflect differential membrane lipid composition, posttranslational modifications, or interactions of VR1 with other cellular components in mammalian versus amphibian cells.

In membrane patches excised from VR1-transfected (but not vector-transfected) HEK 293 cells, protons evoked discrete single channel openings (Figure 4D). These responses exhibited a unitary amplitude (at +40 mV) similar to that observed for heat-evoked and capsaicin-evoked responses. Interestingly, acidified bath solution elicited responses only when applied to outside-out membrane patches, suggesting that protons interact with a region of the receptor that resides on the extracellular face of the plasma membrane. Candidate sites for proton interaction include several acidic residues found within putative extracellular loops of the VR1 protein. Neutralization of these residues by protonation might alter VR1 function by destabilizing hydrophilic interactions and/or stabilizing hydrophobic interactions that favor ion channel opening. Taken together, our observations demonstrate that, in mammalian cells, extracellular protons activate VR1 in the absence of cytoplasmic components and in a concentration range readily achieved during tissue injury.

Distribution of VR1 Protein

Capsaicin sensitivity is probably the best functional marker of C-fiber nociceptors (Jancso et al., 1977; Martin et al., 1987; Szolcsanyi et al., 1988), and, indeed, VR1 transcripts are found exclusively in small to medium diameter primary sensory neurons (Caterina et al., 1997). Antibodies to the VR1 protein would therefore provide an important molecular marker for these cells and facilitate the anatomical analysis of the primary afferent component of the "pain" pathway. We therefore raised a rabbit antiserum against a synthetic peptide corresponding to the predicted VR1 carboxyl terminus. Immunoblot analysis of whole-cell lysates from VR1-transfected HEK 293 cells revealed a tight doublet migrating at ~90 kDa, close to the predicted molecular mass of VR1 (Figure 5F). This doublet was not observed in lysates from vector-transfected control cells. Addition of immunizing peptide eliminated the doublet, whereas addition of an unrelated peptide had no effect, confirming the specificity of the antiserum.

Our immunocytochemical analysis focused on the distribution of VR1 in the spinal cord and caudal brainstem, where primary sensory neurons send their central projections from dorsal root and trigeminal ganglia, respectively (Marfurt, 1981; Molander and Grant, 1985). In general, the pattern of immunostaining was comparable to that observed when the capsaicin receptor was localized by binding of [^{3}H]-resiniferatoxin (Winter et al., 1993; Acs et al., 1994; Szallasi et al., 1995). Intense immunoreactivity was observed in the terminals of afferent fibers projecting to the superficial layers of the spinal cord dorsal horn (Figures 5A and 5C) and the trigeminal nucleus caudalis (Figure 5B). In the spinal cord, the densest staining was found in laminae I and II, although some labeled axons extended ventrally to the neck of the dorsal horn and around the central canal (lamina V and X, respectively). Projections to laminae V and X arose from axons that coursed along the medial edge of the dorsal horn or from small bundles of axons that penetrated the superficial dorsal horn. These patterns distinguish the VR1-expressing axons from many Aδ afferents that reach the neck of the dorsal horn after arborizing around the lateral edge of the dorsal horn (Light and Perl, 1979). The latter observation is of interest because some studies have suggested that Aδ nociceptive fibers are also sensitive to capsaicin (Nagy et al., 1983).

The pattern of staining in the sacral spinal cord differed considerably from that observed at other spinal levels (Figure 5C). Specifically, here we found extensive axonal staining throughout the dorsal horn. Some fibers

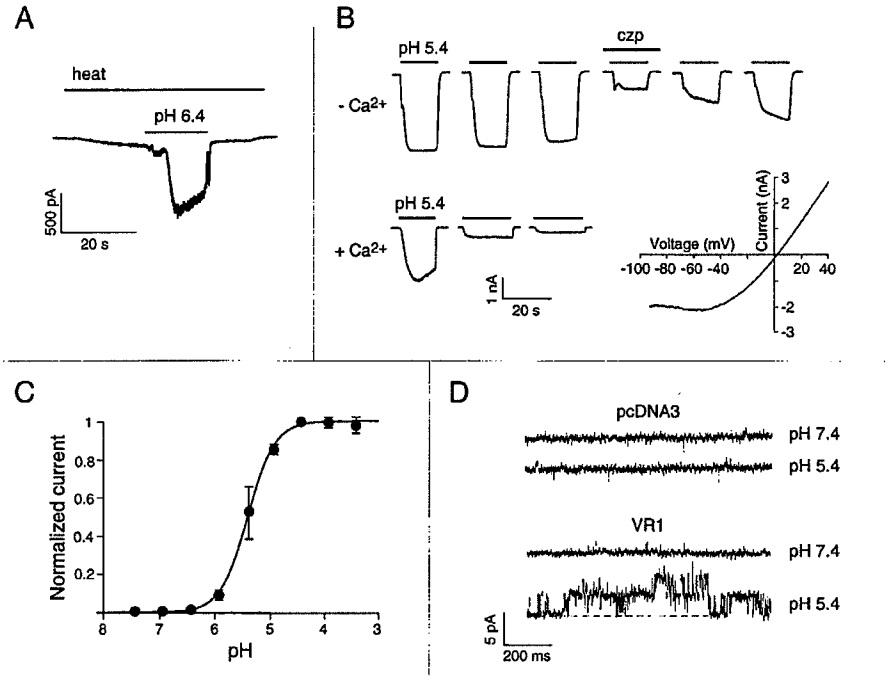

Figure 4. Protons Activate VR1 at Normal Physiological Temperature in HEK 293 Cells

(A) pH 6.4 bath solution activated whole-cell currents in VR1-transfected cells (−60 mV) at 37°C.

(B) High concentrations of protons at 22°C evoked currents in VR1-transfected cells that resemble capsaicin-evoked responses. Under Ca^{2+}-free conditions (top), protons evoked nondesensitizing responses that were reversibly blocked (78.9% ± 4.1%, n = 4) by capsazepine (czp, 10 μM). In Ca^{2+}-containing solution (bottom), proton-evoked currents showed profound desensitization. Whole-cell current–voltage curve generated by a ramp pulse (−100 to +40 mV in 500 ms) showed pronounced outward rectification. Acidic bath solution was applied at 2 min intervals, and pretreatment time with capsazepine was 2 min.

(C) Concentration–response curve for protons shows half-maximal response at pH 5.4. Normalized whole-cell currents (mean ± SEM, n = 12) are expressed as a fraction of pH 4.4 bath solution responses. The Hill equation was used for curve fitting.

(D) Protons (pH 5.4) produce single channel openings in outside-out patches excised from VR1-expressing cells but not vector-transfected cells (E_h = +40 mV). Broken line indicates closed channel level. Mean unitary amplitude was 2.80 ± 0.08 pA (n = 8). No inside-out patches showed responses to acidified bath solution (n = 8). Of these, five were tested for capsaicin responses, and all were positive.

projected to the neck of the dorsal horn, while others could be traced from the dorsal root entry zone to lamina X and thence to the opposite side of the spinal cord. This pattern of staining is reminiscent of the arborization of small diameter pelvic visceral afferents (Morgan et al., 1981). Consistent with this assignment, we observed dense VR1 immunoreactivity in the nucleus of the solitary tract and area postrema, medullary structures that receive vagal projections from visceral organs via the nodose ganglion (Figure 5D). Although we previously failed to detect VR1 messenger RNA within the nodose ganglion (Caterina et al., 1997), the present results clearly demonstrate the expression of this protein within the central targets of vagal afferents. We also observed VR1 immunoreactivity in the vagus nerve, peripheral to the nodose ganglion (data not shown) and in tortuous nerve terminals within muscular and submucosal layers of the bladder (Figure 5E). These findings are consistent with previous reports showing capsaicin sensitivity and [^{3}H]-resiniferatoxin labeling of cells in the nodose ganglion (Szallasi et al., 1995) and marked sensitivity of bladder afferents to capsaicin (Maggi, 1992). VR1 immunoreactivity was also observed in peripheral projections of sensory neurons. Although some receptors can only be detected in the peripheral nerve after ligation (to build up the antigen level at the ligature) (Liu et al., 1994), we found considerable VR1 staining along the course of the sciatic nerve (i.e., very distant from the terminals), suggesting either that the receptor is inserted along the course of the peripheral nerve membrane or that relatively large quantities are transported intraaxonally. By contrast, we found no staining of sympathetic postganglionic neurons, nor did we observe staining in central neurons in the brain, including the preoptic area of the hypothalamus, which reportedly has some resiniferatoxin binding (Acs et al., 1996) and responsiveness to vanilloids (Ritter and Dinh, 1992; Szolcsanyi, 1993).

Electron microscopic (EM) analysis of spinal cord sections revealed VR1 immunoreactivity in large numbers of unmyelinated axons in the dorsal horn (Figure 6A). Importantly, even the rare axon that penetrated into lamina III proved by EM to be unmyelinated. To date, we have not observed labeling of myelinated axons; however, this result could reflect incomplete antibody penetration. VR1 immunoreactivity was also found at nerve terminals, where it was typically concentrated away from synaptic specializations and from regions occupied by collections of clear vesicles (Figure 6B). In some cases, the immunostaining was associated with dense core vesicles, but our use of an enzyme-coupled (DAB)

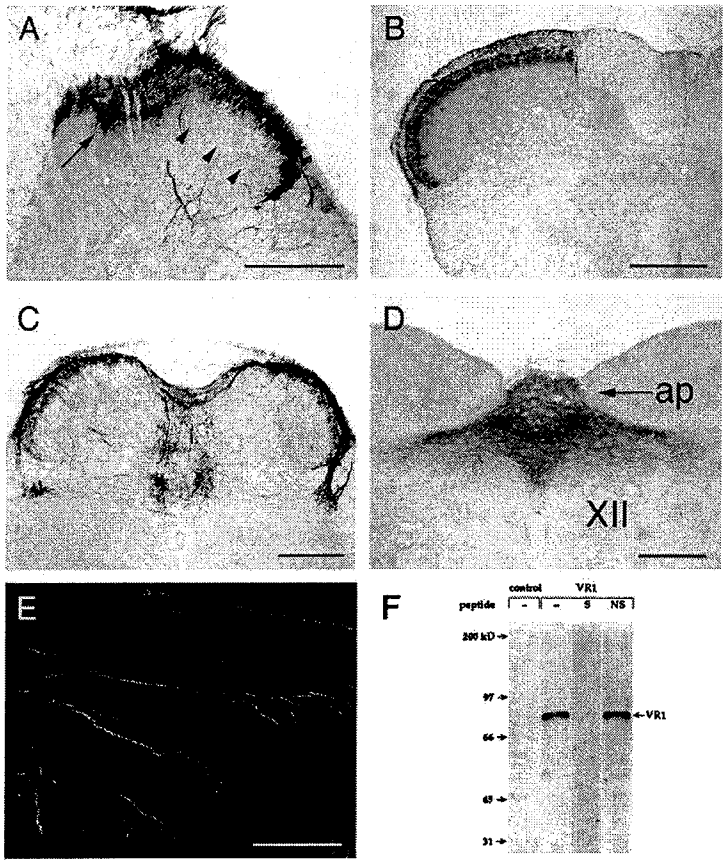

Figure 5. VR1 Is Located in Somatic and Visceral Primary Afferent Neurons

(A–E) Immunoperoxidase staining reveals intense VR1 immunoreactivity in lumbar spinal cord dorsal horn (A), trigeminal nucleus caudalis (B), sacral spinal cord dorsal horn (C), and nucleus of the solitary tract and area postrema (ap) of the caudal medulla (D). Note the characteristic patch of intense VR1 immunoreactivity in the medial part of inner lamina II (arrow), with much less VR1 immunoreactivity in the lateral part of inner lamina II (arrowheads; also see Figure 7E). Immunofluorescence staining reveals VR1 immunoreactivity in axons that arborize in the bladder (E) as well as in the vagus and sciatic nerves (data not shown). Scale bars, 200 μm (A and C); 375 μm (B); 100 μm (D); and 500 μm (E).

(F) Immunoblot analysis reveals a specific 90 kDa band in lysates from VR1-transfected HEK 293 cells but not from pcDNA3-transfected control cells. This band was eliminated by preincubation with the peptide used to generate the VR1 antiserum (S) but not with control peptide (NS). The band was not detected using preimmune serum (data not shown).

detection method did not provide the resolution necessary to establish this point unequivocally.

In the adult rodent, primary afferent nociceptors have been largely divided into two histochemically distinct classes: one expresses neuropeptides (e.g., substance P and calcitonin gene-related peptide [CGRP]); the other expresses specific enzyme markers (e.g., fluoride-resistant acid phosphatase) and binds the lectin IB4. These two classes of neurons are sensitive to the neurotrophic factors nerve growth factor (NGF) and glial cell line–derived neurotrophic factor (GDNF), respectively (Snider and McMahon, 1998). Although sensitivity to neonatal treatment with capsaicin has been observed in both populations (Hammond and Ruda, 1991), the extent to which this is maintained in the adult and mediated through VR1 is unclear. To address these issues, we carried out colocalization studies of IB4 binding and VR1 and substance P immunoreactivity in DRG and spinal cord dorsal horn. In lumbar DRG, we found that most (~85%) of the substance P–immunoreactive cells costained for VR1 (Figure 7A). The much larger population of IB4-positive cell bodies also immunostained for the VR1 but to a lesser extent (60%–80%) (Figure 7B). Finally, we found that ~10% of the VR1-positive neurons stained for neither substance P nor IB4. Similar patterns of costaining were observed in the superficial dorsal horn of the spinal cord (Figure 7E). Here, we found extensive double labeling of substance P and VR1 in lamina I and the outer portion of lamina II, where most peptide-containing afferents terminate. As expected, we found dense IB4 labeling in the inner part of lamina II (IIi), but, interestingly, we found colocalization of dense VR1 immunoreactivity and IB4 binding only in the medial part of IIi. The lateral part of IIi only stained for IB4 (see also Figure 5A). This differential colocalization reveals a heretofore undetected heterogeneity of the small diameter primary afferent termination within the inner portion of lamina II. Alternatively, these axons may derive from DRG neurons that express VR1 but do not target the receptor to the central terminal.

Discussion

VR1 is a Polymodal Signal Detector

We have shown that the cloned capsaicin receptor can be activated by several stimuli, including vanilloid compounds, heat, and protons. Is VR1, by itself, sufficient to mediate responses to these stimuli? The involvement of additional proteins or other cellular components cannot be formally ruled out until active channel protein has been studied in a homogeneous and defined preparation. Nevertheless, because activation or modulation can occur dynamically, in excised membrane patches, and in different cellular environments, we suggest that the ability of VR1 to respond to these dissimilar agonists is an intrinsic property of the channel protein.

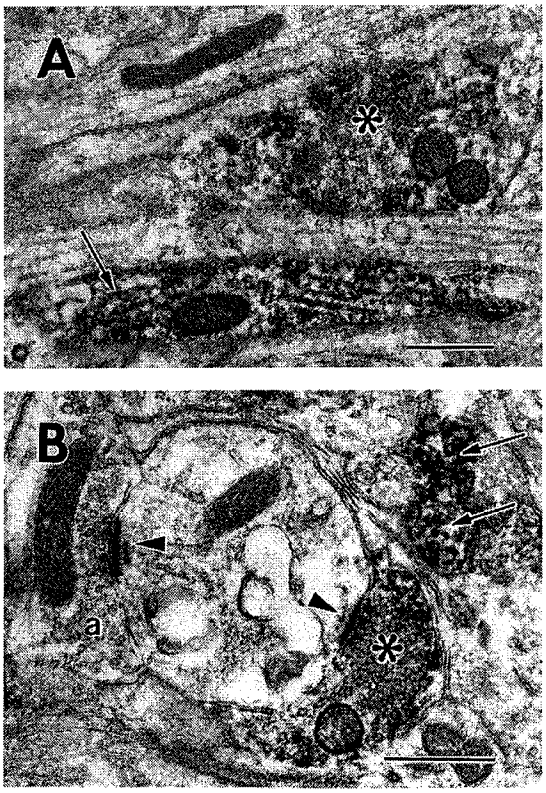

Figure 6. VR1 Immunoreactivity Is Concentrated in Synaptic Terminals in the Dorsal Horn

(A) A sagittal section reveals VR1 immunoreactivity in an unmyelinated axon and a synaptic bouton. Axonal labeling is associated with microtubules (arrow) as well as with the plasma membrane.

(B) VR1 immunoreactivity is seen in a terminal that is presynaptic to a dendrite receiving convergent input from a VR1-negative terminal (a). In the synaptic terminal, VR1 immunoreaction product is often located away from the clusters of clear synaptic vesicles (asterisks in [A] and [B]). The presence of the synaptic density (arrowhead in [B]) makes it difficult to determine whether there is VR1 immunoreactivity at the active zone. Scale bars, 0.2 μm.

How do different VR1 agonists promote channel opening and to what extent do their gating mechanisms overlap? It appears that heat, protons, and vanilloids do not interact with the channel in completely equivalent ways. For example, although both capsaicin and proton-induced desensitization are calcium dependent, heat-induced desensitization is independent of extracellular calcium. In addition, the ability of heat and capsaicin to overcome desensitization when applied together, but not separately, suggests that the effects of these agonists on the channel differ. Together, these findings argue against a simple model in which all three agonists act at one site to gate channel opening through a single conserved mechanism. Perhaps this is to be expected, since heat and protons, in contrast with drugs, will have global effects on protein conformation and membrane properties that could influence the nature of ion channel responses to these stimuli. On the other hand, because capsazepine blocks capsaicin-, heat-, and proton-evoked currents, there is clearly some degree of convergence in the mechanisms of action of these three stimuli. Assuming that capsazepine blocks responses to all three agonists via the same mechanism, this observation rules out another extreme model wherein completely nonconvergent pathways lead to channel activation.

An alternative and particularly intriguing model is one in which capsaicin binding alters the sensitivity of VR1 to temperature and acidity such that the channel opens under normal physiological conditions. In this scenario, capsaicin is not an agonist per se but functions as a modulatory agent, lowering the channel's response threshold to the ubiquitous actions of heat and protons. Precedence for such a mechanism is provided by two other lipid-soluble plant-derived toxins, aconitine and veratridine. These alkaloid compounds act as "agonists" for voltage-gated sodium channels by altering their voltage dependence so that the channel opens at resting membrane potential (Strichartz et al., 1987; Hille, 1992). Interestingly, aconitine and veratridine reduce the strong preference that voltage-gated Na^+ channels exhibit for Na^+ ions, a phenomenon that resembles the quantitative differences we observe in the relative ionic permeability ratios of heat- and capsaicin-evoked responses. This model highlights the notion that vanilloids, heat, and protons act in concert to regulate VR1 activity; the effects of any one stimulus cannot be considered in isolation. Mutational analysis of VR1 function and the development of stimulus-specific VR1 antagonists will help to distinguish among these and other potential mechanisms of channel activation.

VR1 As a Mediator of Sustained Proton Responses In Vivo

In sensory neurons, proton-evoked currents consist of two major components: one is rapidly inactivating and Na^+ selective with a linear current–voltage relation (Krishtal and Pidoplichko, 1980; Konnerth et al., 1987); the other is a more sustained, nonselective cation conductance with an outwardly rectifying current–voltage profile (Bevan and Yeats, 1991; Bevan and Docherty, 1993; Bevan and Geppetti, 1994). The latter component is believed to underlie the prolonged sensation of pain that accompanies tissue acidification. Although it has been proposed that DRASIC, a member of the degenerin/amiloride-sensitive cation channel family (Waldmann et al., 1997b) mediates the sustained phase of proton-evoked responses in sensory neurons, the pH dependence of the sustained component of DRASIC-mediated currents (half-maximal effective $pH_o = 3.5$) is significantly lower than that of sustained proton-evoked currents in sensory neurons. VR1, by contrast, is activated by protons in a concentration range that more closely resembles that associated with sustained proton-evoked currents in sensory neurons (Bevan and Yeats, 1991; Kress et al., 1996), proton-stimulated peptide release from innervated tissue (Geppetti et al., 1990; Bevan and Geppetti, 1994), and ischemic and inflammatory models of tissue injury (Jacobus et al., 1977; Steen et al., 1992; Bevan and Geppetti, 1994). Despite this similarity, it is still uncertain whether VR1 can fully account for the pharmacological properties of sustained

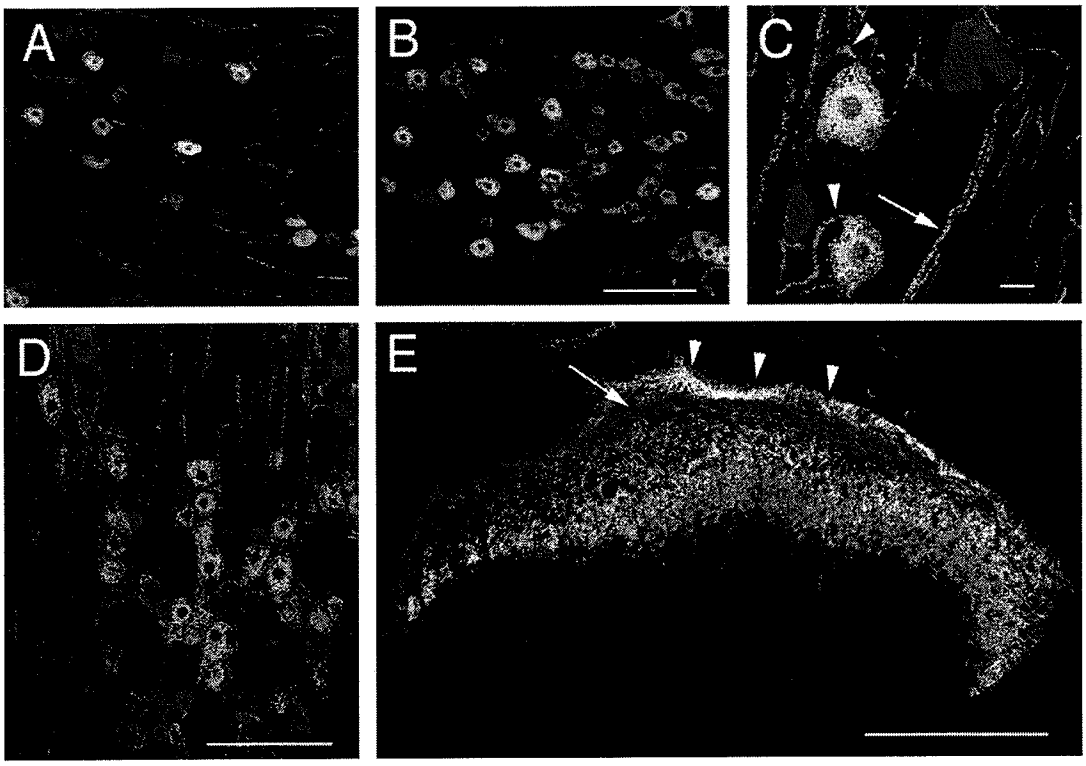

Figure 7. VR1 Is Expressed in Both Peptide-Containing and Nonpeptide Primary Afferent Neurons

Double immunofluorescence was used to colocalize VR1 (red) and substance P (green) immunoreactivity (A and C) or VR1 immunoreactivity (red) and lectin IB4 binding (green) (B) in DRG cell bodies. The great majority of substance P–immunoreactive cell bodies also express VR1 (yellow in [A] and [C]); however, although many of the axons are also double labeled (arrow in [C]), some axons of substance P/VR1-positive neurons were only substance P positive (arrowheads in [C]). Numerous IB4/VR1-positive cell bodies were found in the DRG, but many IB4-positive neurons do not express VR1 (green cells in [B]). Triple labeling for VR1 (red), substance P (blue), and IB4 (green) in the DRG (D) reveals the presence of VR1-expressing neurons that are neither substance P nor IB4 positive. Triple labeling in the dorsal horn (E) reveals extensive terminal overlap of VR1 and substance P in laminae I and outer II (purple). There appears to be some VR1-only labeling in lamina I (arrow). Intense double labeling of VR1 and IB4 is seen in axons of the Lissauer tract (arrowheads) and in the medial part of inner lamina II (yellow); however, there is also a dense concentration of IB4-only axons (green) in the lateral part of inner lamina II. Scale bars, 50 μm (B, D, and E); 10 μm (C).

proton-evoked pain responses. Most notably, although some studies have reported capsazepine block of proton-evoked responses in sensory neurons (Santicioli et al., 1993; Liu and Simon, 1994; Fox et al., 1995), others have not (Bevan et al., 1992; Bevan and Geppetti, 1994; Fox et al., 1995). These discrepancies could reflect functional redundancy of proton-sensing molecules, differences in experimental protocols, or nonselective and noncompetitive mechanisms of capsazepine action (Liu and Simon, 1997). Nevertheless, VR1 remains a viable candidate for a transducer of proton-evoked responses in the "pain" pathway.

VR1 Localization and Nociceptor Heterogeneity

A recent review on the molecular biology of nociceptors raised questions regarding the expression of VR1 in peptide-containing, NGF-sensitive sensory neurons versus IB4-binding, GDNF-sensitive neurons (Snider and McMahon, 1998). Our demonstration of VR1 immunoreactivity within substance P–positive and IB4-positive populations suggests that VR1 can account for the capsaicin sensitivity of both cell types. At the same time, our finding that many IB4-positive cells were not VR1 positive provides evidence for the existence of two neurochemically distinct populations of IB4-binding nociceptors that project to anatomically discrete regions of lamina II of the spinal cord dorsal horn. Because the medial and lateral regions of the lumbar dorsal horn represent distal and proximal parts of the hindlimb, respectively (Devor and Claman, 1980), this result further suggests that functionally distinct afferent subtypes innervate topographically different regions of the hindlimb. Finally, these results are consistent with there being a subpopulation of IB4-positive cells that is either resistant to capsaicin action or sensitive by virtue of another receptor subtype. Given that VR1 responds to thermal and chemical stimuli and that the traditional polymodal nociceptor also responds to noxious mechanical stimuli (Bessou and Perl, 1969), it will be of great interest to determine whether mechanical sensitivity is differentially manifest within the nociceptor populations described.

Polymodal Activation of VR1 In Vivo

The relative importance of noxious thermal and chemical stimuli as activators of VR1 in vivo is likely to vary with

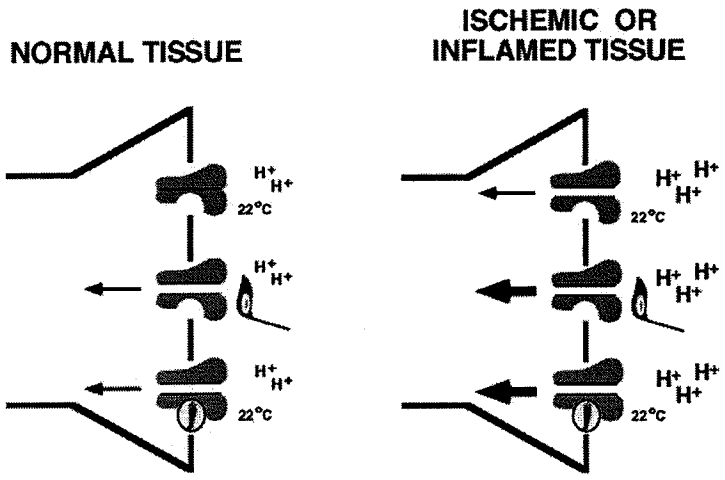

Figure 8. Proposed Model for VR1 Integration of Painful Stimuli in Normal and Ischemic or Inflamed Tissues

Heat (flame) or capsaicin (pepper) can evoke pain in normal animals by activating VR1 in nociceptor terminals at physiological pH (left). In ischemic and inflamed tissue (right), we hypothesize that VR1 is activated at room temperature by the elevated proton (H^+) concentrations that these insults produce. In addition, protons potentiate responses evoked by heat or capsaicin, resulting in increased nociceptor activity. Thickness of arrows corresponds to relative magnitude of nociceptor activity, which in turn contributes to the magnitude of the resultant pain response.

the anatomical location and nature of the insult. Thus, in superficial (e.g., cutaneous) tissues VR1 activation might result from acute elevations in temperature or reductions in pH, whereas in visceral or deep somatic tissues (where temperatures are unlikely to reach the "noxious" level of 43°C) changes in pH are more likely to drive VR1 activation. These scenarios do not preclude the existence of other modulators of VR1 activity, such as novel VR1 agonists or proalgesic agents (e.g., bradykinin and prostaglandins) that could alter vanilloid receptor function indirectly via second messenger signaling pathways (Levine and Taiwo, 1994). Moreover, channels other than VR1 may contribute to the thermal and chemical sensitivity of primary afferent neurons.

Following tissue injury, the magnitude of VR1 responses is likely to reflect the combined effects of protons and temperature (Figure 8). We have shown that in the context of a normal ambient thermal stimulus (i.e., 37°C) moderate reductions in pH can activate VR1. This interaction would be expected to facilitate pain responses and/or autonomic reflexes during relatively mild acidosis in ischemic or inflamed tissues. Reductions in pH that accompany tissue injury would also be expected to enhance the responsiveness of those tissues to exogenously applied thermal stimuli. In fact, the augmentation of VR1 thermal responsiveness by protons shows a striking resemblance to the increase in cutaneous nociceptor thermal sensitivity associated with inflammation (Fields, 1987; Dubner and Basbaum, 1994; Meyer et al., 1994). In both cases, there is a significant decrease in the threshold for heat-evoked responses and an increase in response magnitudes at temperatures above the initial "pain" threshold. Given these parallels, modulation of VR1 activity may serve as a useful model system with which to analyze the molecular basis of the sensitization of nociceptors that contributes to the development of allodynia and hyperalgesia.

Experimental Procedures

Mammalian Cell Electrophysiology

Patch-clamp recordings were carried out with VR1-transfected HEK 293 cells in whole-cell, inside-out, and outside-out configurations as previously described (Caterina et al., 1997). Standard bath solution for whole-cell recordings contained (in mM) 140 NaCl, 5 KCl, 2 $MgCl_2$, 2 $CaCl_2$, 10 HEPES, and 10 glucose (adjusted to pH 7.4 with NaOH). In Ca^{2+}-free bath solution, $CaCl_2$ was replaced with 5 mM EGTA. Bath solution was buffered to different pH values with either 10 mM HEPES (6.9 and 6.4) or 10 mM MES (5.9, 5.4, 4.9, 4.4, 3.9, and 3.4). Ca^{2+}-free bath solution was used unless otherwise stated. For divalent cation substitution experiments, whole-cell configuration was obtained in standard bath solution, after which the solution was changed to (in mM) 140 NaCl, 10 glucose, and 10 HEPES (adjusted to pH 7.4 with NaOH) followed by 110 $MgCl_2$ (or $CaCl_2$), 2 $Mg(OH)_2$ (or $Ca[OH]_2$), 10 glucose, and 10 HEPES (adjusted to pH 7.4 with HCl). Reversal potential was measured using voltage ramps (−100 to +40 mV in 500 ms). Bath solution for outside-out patch recordings and pipette solution for inside-out patch recordings contained (in mM) 140 NaCl and 10 HEPES (adjusted to pH 7.4 with NaOH). Bath solution for inside-out patch recordings and pipette solutions for outside-out patch recordings and cation substitution experiments contained (in mM) 140 NaCl, 10 HEPES, and 5 EGTA (adjusted to pH 7.4 with NaOH). Pipette solution for other whole-cell recordings contained (in mM) 140 CsCl (or 130 Cs aspartate and 10 NaCl for obtaining the current–voltage curve), 5 EGTA and 10 HEPES (adjusted to pH 7.4 with CsOH). Liquid junction potentials were measured directly in separate experiments and did not exceed 3 mV with solutions used at 22°C. In heat experiments, significant liquid junction potential changes (>6 mV) were observed and corrections made for membrane potentials and reversal potentials. Whole-cell recording data were sampled at 20 kHz and filtered at 5 kHz for analysis (Axopatch 200 amplifier with pCLAMP software, Axon Instruments). Single channel recording data were sampled at 10 kHz and filtered at 2 kHz. Permeability ratios for divalent cations to Na^+ (P_X/P_{Na}) were calculated as previously described (Caterina et al., 1997). Linear and nonlinear regression analyses were conducted using Origin (Microcal) or DeltaGraph (Delta Point).

Oocyte Electrophysiology

Defolliculated *Xenopus laevis* oocytes were injected with 5–10 ng VR1 cRNA as previously described (Caterina et al., 1997). Two-electrode voltage-clamp analysis (E_h = −40 mV) was carried out 4–7 days postinjection. Frog Ringer's solution contained (in mM) 90 NaCl, 1.0 KCl, 2.4 $NaHCO_3$, 0.1 $BaCl_2$, 1.0 $MgCl_2$, and 10 HEPES (at pH 7.6, unless otherwise indicated). Bath solution temperature was controlled with an in-line SH-27A solution heater and TC-324B thermal controller (Warner Instruments) and was accurate to within 0.1°C between 40°C and 50°C. Below 40°C, accuracy decreased with decreasing temperature to a maximum deviation of 1.1°C at 30°C and 2.7°C at 22°C. A thermistor was placed within 1 mm of the oocyte to record bath temperatures and regulate the heat controller.

Immunolocalization of VR1

A peptide encoding the predicted carboxyl terminus of VR1 (EDAEV FKDSMVPGEK) was coupled to keyhole limpet hemocyanin via an

amino-terminal cysteine and used to immunize rabbits (HTI Bioproducts). Crude serum or ammonium sulfate–purified material was used at specified dilutions (Harlow and Lane, 1988). For immunoblot analysis, HEK 293 cells were transiently transfected with 24 μg control vector (pcDNA3) or VR1–pcDNA3, as described (Caterina et al., 1997). After 16 hr, cells were washed with phosphate-buffered saline/1 mM EDTA, lysed with Laemmli sample buffer, and incubated at 55°C for 10 min. Then, 0.5% of this sample was subjected to SDS-PAGE on a 7.5% Laemmli gel, blotted to a nitrocellulose membrane, and probed with anti-VR1 serum (diluted 1000-fold) in the absence or presence of VR1 carboxy-terminal peptide (5 μg/ml). Immunodetection was performed using the enhanced chemiluminescence (ECL) Western blotting kit (Amersham).

For light microscopic studies, male C57BL/6 mice (23–28 g) or male Sprague-Dawley rats (250–300 g) (Bantin-Kingman Laboratories) were deeply anesthetized with 100 mg/kg sodium pentobarbital administered intraperitoneally and perfused through the ascending aorta with 0.1 M phosphate-buffered saline followed by a 10% formalin or 4% paraformaldehyde fixative. After perfusion, tissues were removed, postfixed for 4 hr in the same fixative, and then cryoprotected in 30% sucrose overnight at 4°C. The spinal cord was sectioned transversely at 30 μm on a freezing microtome; DRG, sciatic nerve, and superior cervical ganglion sections (10 μm) and bladder and vagus nerve sections (25 μm) were cut on a cryostat. For immunocytochemistry, sections were incubated overnight in anti-VR1 antiserum diluted 1:50,000. Immunostaining was performed according to the avidin–biotin peroxidase method (Hsu et al., 1981) using a nickel-intensified diaminobenzidine protocol with glucose oxidase to localize the horseradish peroxidase immunoreaction product. Preincubation of the primary antiserum with 10 μg/ml of the VR1 carboxy-terminal peptide abolished staining (data not shown). Reacted sections were mounted on gelatin-coated slides, dried, dehydrated, and then coverslipped with Eukitt (Calibrated Instruments). Sections were examined with a Nikon Microphot-FXA microscope and digitized images obtained with a MicroLumina camera (Leaf Systems); photomontages were created with Adobe Photoshop. To localize VR1 in bladder, sciatic nerve, and vagus nerve, sections were incubated overnight (4°C) with VR1 antiserum (diluted 1:5,000–1:7,500), washed, incubated 2 hr with Cy3-conjugated goat anti-rabbit IgG (Jackson Immunochemicals, diluted 1:600), washed, dehydrated, and mounted in DPX (Electron Microscopy Sciences). Preincubation of the primary antiserum with 10 μg/ml of the VR1 carboxy-terminal peptide abolished immunofluorescent staining, whereas preincubation with a control peptide did not (data not shown). To double label DRG cells for VR1 and substance P, we incubated tissue overnight in the rabbit VR1 antiserum diluted 1:5,000 and in a guinea pig anti-substance P antiserum diluted 1:15,000 (kindly provided by Dr. John Maggio). The two primary antisera were respectively localized with a Cy3-coupled goat-anti rabbit IgG diluted 1:200 and a Cy2-coupled goat anti-guinea pig IgG diluted 1:600 (both from Jackson Immunochemicals). For costaining with *Griffonia simplicifolia* IB4 lectin, sections were incubated with 12.5 μg/ml FITC-conjugated IB4 lectin (Sigma) and then with VR1 antiserum using a Cy3-conjugated secondary antiserum. Reacted sections were examined with a BioRad 1024 confocal microscope (Bio-Rad). Confocal images were transferred to NIH image and then analyzed with Adobe Photoshop.

For electron microscopy, adult rats were perfused with 4% formaldehyde and 1% glutaraldehyde in phosphate buffer, and spinal cords were sectioned on a Vibratome. After treatment of sections with 50% ethanol to enhance penetration of antibodies, we immunostained for VR1 at the EM level as previously described (Llewellyn-Smith and Minson, 1992). Immunoreacted sections were then osmicated, embedded in plastic, and thin sectioned for examination in a JEOL electron microscope.

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EXHIBIT C

The vanilloid receptor TRPV1: 10 years from channel cloning to antagonist proof-of-concept

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Abstract | The clinical use of TRPV1 (transient receptor potential vanilloid subfamily, member 1; also known as VR1) antagonists is based on the concept that endogenous agonists acting on TRPV1 might provide a major contribution to certain pain conditions. Indeed, a number of small-molecule TRPV1 antagonists are already undergoing Phase I/II clinical trials for the indications of chronic inflammatory pain and migraine. Moreover, animal models suggest a therapeutic value for TRPV1 antagonists in the treatment of other types of pain, including pain from cancer. We argue that TRPV1 antagonists alone or in conjunction with other analgesics will improve the quality of life of people with migraine, chronic intractable pain secondary to cancer, AIDS or diabetes. Moreover, emerging data indicate that TRPV1 antagonists could also be useful in treating disorders other than pain, such as urinary urge incontinence, chronic cough and irritable bowel syndrome. The lack of effective drugs for treating many of these conditions highlights the need for further investigation into the therapeutic potential of TRPV1 antagonists.

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Ten years ago in 1997, the field of somatic sensory biology and pain research witnessed the breakthrough work of David Julius and colleagues that led to the cloning of the first vanilloid (capsaicin) receptor, transient receptor potential vanilloid subfamily, member 1 (TRPV1)[1]. Capsaicin, which is responsible for the piquancy of hot-chilli peppers, is a versatile natural compound, the biological use of which is covered by more than 900 patents. Uses range from food flavouring and bird seeds (included to repel squirrels) to pepper spray for self-defence and ointments for the relief of neuropathic pain[2]. Capsaicin is unique among naturally occurring irritant compounds in that the initial neuronal excitation that it evokes is followed by a durable refractory state during which the previously excited neurons are unresponsive to a broad range of seemingly unrelated stimuli[2]. This effect, traditionally referred to as desensitization, has a clear therapeutic potential. In fact, capsaicin-containing creams have been in clinical use for decades to relieve painful conditions such as diabetic neuropathy[3].

Generally speaking, capsaicin-sensitive neurons are bipolar neurons with unmyelinated axons (C-fibres) and somata in sensory (dorsal root and trigeminal) ganglia[4]. Of note, a subset of sensory neurons with thin myelinated axons (Aδ fibres) is also capsaicin sensitive[4]. As discussed later, the expression of TRPV1 in Aδ fibres is upregulated during nerve-injury-induced thermal hyperalgesia[5] and diabetic neuropathy[6], which makes TRPV1 an important target for pain relief. The peripheral termini of capsaicin-sensitive neurons are sites of release for various pro-inflammatory neuropeptides such as substance P (SP) and calcitonin gene-related peptide (CGRP) that, in turn, initiate the biochemical cascade collectively known as neurogenic inflammation[7,8]. Disease states that have a significant neurogenic inflammatory component include migraine, asthma, inflammatory bowel disease (IBD), interstitial cystitis and osteoarthritis (reviewed in REF. 7). The central fibres of capsaicin-sensitive neurons enter the dorsal horn of the spinal cord where they form synapses with second-order neurons[2,4]. The central role of TRPV1 in the initiation of the neurogenic inflammatory response and the transduction of pain is well established[2–4,7,8]. Of note (as reviewed in REFS 9,10), TRPV1 is also present in brain nuclei and non-neuronal tissues. As to the biological roles of TRPV1 receptors in these tissues, speculations are abundant, but conclusive evidence is still absent.

The cloning of TRPV1 represented a significant step in our understanding of the molecular mechanisms that underlie the transduction of noxious thermal and

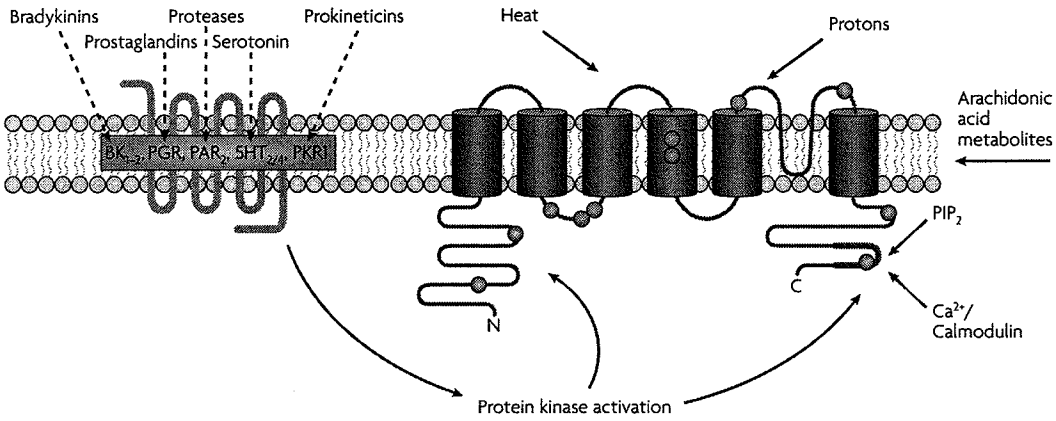


Figure 1 | **Schematic summary of TRPV1 signal integration in the peripheral nociceptor terminal.** Solid arrows indicate transient receptor potential vanilloid subfamily, member 1 (TRPV1)-sensitizing stimuli. The red arrows indicate negative regulation by phosphatidylinositol 4,5-bisphosphate (PIP_2), calcium and calmodulin. Receptors and cognate ligands known to mediate the sensitization of TRPV1 are shown on the left. These largely sensitize TRPV1 through protein-kinase activation, although increased arachidonic acid metabolite production and PIP_2 hydrolysis are also important. Coloured circles represent amino-acid residues that have been identified to be important in particular functions: orange, vanilloid binding (Y511, S512, L547, T550); blue, protein kinase phosphorylation sites (S116, T370, S502, T704, S800); and green, low-pH activation (E600, E646). The red line indicates the carboxy-terminal domain of TRPV1, which has been shown to interact with both PIP_2 and calmodulin.

chemical stimuli by sensory neurons[1]. TRPV1 is now recognized as a molecular integrator of inflammatory mediators (FIG. 1). Consistent with this hypothesis, TRPV1-homozygous-null mice (knockouts) are devoid of thermal hypersensitivity that occurs in response to an acute hind-paw injection of pro-inflammatory agents (for example, complete Freund's adjuvant; CFA), which suggests a clinical value for TRPV1 antagonists as novel analgesic drugs[11,12]. A substantial investment of resources by pharmaceutical companies has led to the discovery of an array of potent and selective small-molecule TRPV1 antagonists, some of which are already undergoing clinical trials (TABLE 1). TRPV1 antagonists are of great interest in that they represent a new strategy in pain relief, because unlike traditional analgesic agents that block the inflammatory response and the propagation and transmission of pain, TRPV1 antagonists aim to prevent pain by blocking an important sensor (transducer) of noxious stimuli on polymodal sensory neurons. The clinical value of TRPV1 antagonists might be the litmus test for the feasibility of this novel approach.

In this Review, we attempt to give a summary of the key characteristics of TRPV1, an overview of the intriguing clinical findings with TRPV1 agonists and a critical assessment of the potential therapeutic effects of the TRPV1 antagonists. We also briefly discuss the salient features of other thermo transient-receptor-potential (thermoTRP) channels[13,14] that are relevant to the pharmacology of TRPV1-expressing cells. TRPV1 is the most studied and validated TRP channel among the thermoTRP class[15], nonetheless, there is mounting evidence to suggest that channels of the TRP family might be the next generation of ion-channel targets that are involved in inflammatory pain[14–16].

The molecular pharmacology of TRPV1

TRPV1, similar to other TRP channels, is a putative six-transmembrane-spanning protein with a pore region localized between transmembrane segments 5 and 6 (REFS 1,17). Consistent with a role in nociception, TRPV1 is a non-selective cation channel with a preference for calcium that is directly activated by capsaicin and noxious temperatures — with an activation threshold *in vitro* of approximately 43°C (REF. 1). These data suggest that TRPV1 might be inactive at a normal body temperature. However, TRPV1 is an exceptional channel in that it is a polymodal nociceptor exhibiting a dynamic threshold of activation that could be significantly lowered under inflammatory conditions (FIG. 1). TRPV1 is thought to mediate the phenomenon of peripheral sensitization that involves a reduction in the threshold of activation and an increase in the responsiveness of the peripheral termini of nociceptors[16]. Indeed, agents in the 'inflammatory soup' act together to lower the activation threshold of TRPV1 (FIG. 1). The growing list of agents that can activate and/or sensitize TRPV1 include: mild acidification[18,19]; bradykinin (an endogenous inflammatory peptide that causes hyperalgesia)[20,21]; nerve-growth factor[21]; anandamide (arachidonoylethanolamide)[22]; arachidonic acid metabolites such as *N*-arachidonoyl-dopamine (NADA, structure shown in FIG. 2) and *N*-oleoyldopamine[23]; lipoxygenase products (12-hydroperoxyeicosatetraenoic acid (12-HPETE) and 15-HPETE)[24]; leukotriene B_4 (REF. 25); prostaglandins[26]; adenosine and ATP[27]; prokineticins[28]; polyamines (such as spermine, spermidine and putrescine)[29]; and venoms from jellyfish[30] and spiders[31].

The mechanism by which TRPV1 integrates such diverse inputs as protons, heat and capsaicin (structure shown in FIG. 2) has important implications for drug

Table 1 | **Current clinical status of TRPV1-targeted therapies**

Therapy name	Compound	Company	Action	Route(s)	Indication(s)	Clinical stage
Transacin	Capsaicin	NeurogesX	Agonist	Transdermal patch	HIV neuropathy-associated pain	Phase III
WL-1001 WL-1002	Civamide (cis-capsaicin)	Winston Laboratories	Agonist	Intranasal, topical	Cluster headache, migraine, osteoarthritis pain	Phase III (headache, osteoarthritis) Phase II (migraine)
ALGRX4975	Capsaicin	Anesiva	Agonist	Injection	Pain	Phase II
SB-705498	SB-705498	GlaxoSmith-Kline	Antagonist	Oral	Migraine, dental pain	Phase II* (migraine) Phase I (dental pain)
NGD 8243	NGD 8243	Neurogen/ Merck	Antagonist	Oral	Pain	Phase II
AMG 517	AMG 517	Amgen	Antagonist	Oral	Pain	Phase I
GRC 6211	GRC 6211	Glenmark	Antagonist	Oral	Osteoarthritis pain, dental pain, incontinence, neuropathic pain	Phase I

*This compound is no longer listed in the GlaxoSmithKline pipeline for migraine. TRPV1, transient receptor potential vanilloid subfamily, member 1.

development. Generally speaking, protein-kinase-dependent phosphorylation of TRPV1 causes sensitization (for example, protein kinase A (PKA)[32] and C (PKC)[33,34]), whereas dephosphorylation by protein phosphatases promotes desensitization[35,36]. Well-described analgesic agents can affect these pathways. For example, morphine blocks TRPV1 sensitization by preventing its phosphorylation by PKA and the synthetic cannabinoid WIN 55,212-2 inhibits TRPV1 through calcineurin-mediated receptor protein dephosphorylation[37,38]. These findings are physiologically relevant as cannabinoid receptor CB1 and δ-opioid receptors are co-expressed with TRPV1 on sensory fibres[39,40]. Furthermore, there is preliminary evidence that suggests that the phosphorylation state of TRPV1 might be disease specific[41]. If so, it might have important implications for drug development as the pharmacological activity of some compounds are affected by the phosphorylation state of TRPV1 (REF. 42).

The capsaicin-binding domain in TRPV1 was first reported by Julius and co-workers in 2002 (REF. 43). Defining the key residues in TRPV1 that are responsible for mediating agonist versus antagonist activity is expected to aid the development of clinically useful ligands. This notion is emphasized by the finding that a single amino-acid mutation (S512Y, located in the intracellular loop between transmembrane domains 2 and 3) converts the potent TRPV1 antagonist iodoresiniferatoxin (I-RTX) into an intrinsic agonist[44]. Resiniferatoxin (RTX, structure shown in FIG. 2) is an ultrapotent capsaicin analogue isolated from the latex of the perennial *Euphorbia resinifera Berg*[2]. High affinity [^{3}H] RTX binding has been linked to S512Y and to other critical residues (in particular, residue 547) in transmembrane domains 3 and 4 (REF. 44). A third domain that is involved in capsaicin gating, but not heat or proton activation, was localized to the pore region of TRPV1 (REFS 44,45). Based on these findings a new model of TRPV1 proposes a 'paddle structure' in which the transmembrane 3 and 4 regions form a gating paddle and residues such as 547 constitute an intracellular vanilloid-binding site[44].

Extracellular amino acids located near the pore domain (FIG. 1) have been implicated in the pH sensitivity of TRPV1 (REF. 46). Recent studies demonstrate that the rabbit anti-rat TRPV1 polyclonal antibody 156H (raised with a synthetic peptide corresponding to the pre-pore loop, E600 to P623) acts as a full antagonist of proton activation but only as a partial antagonist of capsaicin, anandamide and heat activation[47]. It was proposed that antibody 156H locks the channel conformation in the closed (non-conducting) state. Recently, it has been shown that it is the C-terminal domain of TRPV1 that confers heat sensitivity to the channel[48].

It has been postulated that TRPV1 antagonists fall into two categories: class A antagonists, which block the effects of both capsaicin and protons, such as SB-705498, and class B compounds, which are more selective for capsaicin, such as capsazepine[49] (FIG. 2). In addition to sensitization, TRPV1 exhibits agonist-induced channel desensitization that should be distinguished from defunctionalization of the whole neuron by TRPV1 agonists[50]. This is of importance when trying to extrapolate findings in animals 'desensitized to capsaicin' to studies that used antagonists for blockade of TRPV1.

TRPV1: splice variants and related channels

Scant but provocative evidence suggests the existence of TRPV1 splice variants in various tissues. The first rat TRPV1 splice variant to be cloned[51], named stretch-inactivated channel, is also the most controversial in that it appears to be derived from two related but independent genes, raising speculations about its authenticity[52]. The first bona fide TRPV1 splice variant, TRPV1 5′ (originally described as VR1 5′sv), lacks the first approximately 0.5 kb of canonical rat TRPV1

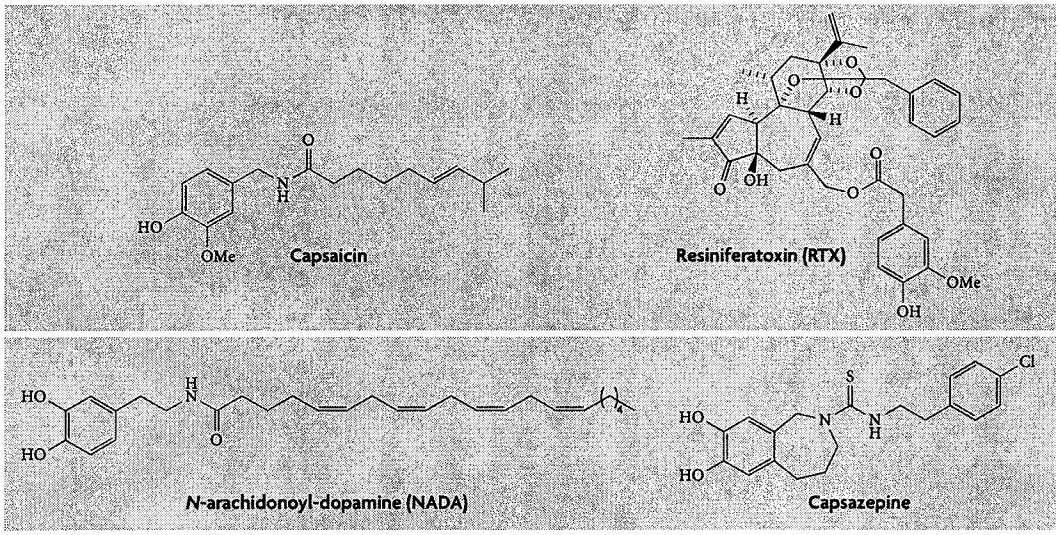


Figure 2 | **Chemical structures of selected TRPV1 ligands.** Capsaicin, the pungent ingredient in hot-chilli peppers; resiniferatoxin, an ultrapotent capsaicin analogue isolated from the cactus-like plant *Euphorbia resinifera Berg*; *N*-arachidonoyl-dopamine, an endogenous lipid mediator in brain nuclei; and the first generation transient receptor potential vanilloid subfamily, member 1 (TRPV1) antagonist, capsazepine.

sequence and is detectable in dorsal root ganglia (where its expression is 12-fold lower than that of TRPV1) and the CNS (at levels comparable to those of TRPV1)[53]. When heterogeneously expressed, TRPV1 5′ is not responsive to vanilloid agonists and the biological role of endogenous TRPV1 5′ is unknown[54]. The recently identified $TRPV1_{VAR}$ is believed to represent a truncated form of the canonical TRPV1 that is present at high levels in renal papillary, but not medullary, lysates[55]. Similar to TRPV1 5′, $TRPV1_{VAR}$ is non-functional[55]. However, when co-expressed with TRPV1, $TRPV1_{VAR}$ acts as a dominant negative modulator[55]. Interestingly, the non-functional murine TRPV1 splice variant TRPV1β can also act in a dominant negative manner[56]. The human TRPV1 variant TRPV1B is closely related to the murine TRPV1β; it is, however, activated by both capsaicin and protons[57]. The number of TRPV1 splice variants is expected to grow in the foreseeable future. For instance, in the kidney at least three distinct TRPV1-related transcripts were detected, which were not present in other tissues[55]. Antagonists designed to inhibit TRPV1 may also act on the splice variants but it is too early to speculate about the possible outcome of such an interaction, as the biological roles of TRPV1 splice variants have yet to be delineated.

Unexpectedly, the concept of a TRPV1-related stretch-inhibited channel was rekindled by the recent finding of pronounced serum hyperosmolarity in *TRPV1*$^{-/-}$ mice[58]. Osmosensory supraoptic nucleus neurons apparently express an N-terminal splice variant, but not the full length, TRPV1. Based on these findings it was surmised that *Trpv1* might encode a central component of the putative hypothalamic osmoreceptor[58].

TRPV1 is the founding member of the thermoTRP subfamily of sensory transducers[13,14] (FIG. 3, TABLE 2) that belong to the larger TRP superfamily of cation channels, which were first described in *Drosophila melanogaster*. Ironically, in this populous receptor subfamily, TRPV1 remains the only 'vanilloid receptor' that is a target for capsaicin and other vanilloids. So far, nine thermoTRP channels have been reported to be activated by changes in temperature. Six of them (TRPV1 to TRPV4, TRP subfamily melastatin, member 8 (TRPM8) and TRP subfamily ankyrin, member 1 (TRPA1)) are expressed in the sensory system and/or skin keratinocytes, and their function in thermosensation is well characterized. The thermoTRP subfamily has two major subdivisions, namely heat- and cold-sensitive channels[13]. TRPV1 is the archetypal, noxious heat-sensitive TRP channel — the group also includes TRPV2 (REF. 59), TRPV3 (REF. 60) and TRPV4 (REF. 61). Cold-responsive TRP channels include TRPM8 and TRPA1 (REFS 62–64). TRPM2 (REF. 65), TRPM4 (REF. 66) and TRPM5 (REF. 67) have recently been shown to be modulated by warm temperatures, but their role in thermosensation remains unclear.

Broadly speaking, thermoTRP channels are expressed on specific subsets of sensory neurons (some also in keratinocytes and other non-neuronal tissues) where they respond to chemicals and to a wide range of temperatures, from innocuous cold and warm to painfully hot or cold (BOXES 1,2). The initial identification of TRPV1 as a noxious stimuli transducer and its validation as a promising target against pain have brought tremendous interest in identifying additional TRPV1-related channels. To date, the TRPV subfamily has a total of six members, TRPV1 to TRPV6, of which TRPV1 to TRPV4 are the focus of interest as potential targets for novel analgesic drugs (BOX 1; FIG. 3). The other two channels, TRPV5 and TRPV6, appear to be constitutively active and are thought to have a role in vitamin-D-dependent calcium uptake in the kidney and intestine, respectively[68].

Expression of TRPV1: implications for therapy

TRPV1 RNA and/or protein expression has been described in various discrete cells, but it is most prevalent in sensory neurons[53]. As reviewed elsewhere[15,69], there is mounting evidence that TRPV1 expression is regulated in sensory neurons at the transcriptional and post-transcriptional levels: for instance, elevated TRPV1-protein levels have been observed in animal models of inflammatory hyperalgesia[70]. These findings are in agreement with the increase in TRPV1-like immunoreactivity that is detected in painful human disease conditions such as IBD[71], faecal urgency, irritable bowel syndrome[72], vulvodynia[73] and mastalgia[74]. In other words, TRPV1-protein upregulation may work together with mechanisms of channel sensitization to drive increased nociceptor afferent activity (mediating pain) as well as neurogenic efferent activity effects through SP and CGRP release (which mediate local vascular and inflammatory effects). Surprisingly, a diffuse loss of TRPV1-positive axons was reported in patients with painful peripheral neuropathies[75]. This finding might provide a rationale to explain the disappointing results obtained in some of the clinical trials that used topical capsaicin for the indication of diabetic neuropathy (reviewed in REF. 2).

Until recently, TRPV1-expressing thin myelinated Aδ fibres received little attention as they comprise a minor subpopulation of Aδ fibres[76]. TRPV1 is, however, not only upregulated on thin myelinated primary afferent neurons in mice with diabetic neuropathy[6] but is also ectopically expressed on Aδ fibres during nerve-injury-induced thermal hyperalgesia[5,77]. Consequently, it was suggested that an increased TRPV1 expression on myelinated fibres might contribute to the anti-hyperalgesic effect of topical capsaicin in diabetic neuropathic pain[75]. Of course, this consideration would also apply to TRPV1 antagonists.

Although neuropeptide release from sensory nerve fibre endings is generally viewed as pro-inflammatory[4,7], some data indicate positive effects on sensory fibre activation and/or SP and CGRP release under certain physiological conditions. For example, acute intragastric capsaicin administration was reported to protect against gastric ulcer formation in animal experiments and in healthy volunteers[78,79]. Hence, although it is theoretically possible that desensitizing doses of TRPV1 agonists or acute pharmacological blockade by TRPV1 antagonists might ameliorate pain while exacerbating pathology, little data exist that addresses this possibility directly. Of particular relevance in this regard is the gastrointestinal (GI) tract, which is rich in TRPV1-positive sensory nerves[80] that will be directly exposed to high concentrations of TRPV1 antagonists if they are given orally as analgesic drugs.

The distribution and possible function of TRPV1 in the CNS will be detailed below. It should be noted, however, that TRPV1 is expressed in higher brain structures[81] that are thought to be involved in pain processing, such as the cingulated cortex (reviewed in REF. 82). As elegantly demonstrated by workers at Abbott, who compared the analgesic activity of TRPV1 antagonists with poor versus good CNS penetration, central TRPV1 exposure might be important for broad-spectrum analgesias[83].

TRPV1 antagonists: for pain relief and more

Overall, the role of TRPV1 as a molecular integrator of noxious stimuli and as an initiator of the neurogenic inflammatory response is universally accepted[7,8]. As a result, numerous companies have initiated programmes to identify TRPV1 modulators. Literature and patent searches, and web-site visits identified the following companies as having active preclinical TRPV1 research activities or programmes: GlaxoSmithKline, Neurogen/Merck, Amgen, Novartis, Abbott, AstraZeneca, Johnson & Johnson, Janssen, Bayer, Takeda, Vertex, Pacific Corp., Euro-Celtique, Digital-Biotech, Schwartz, Renovis/Pfizer, Grünenthal, Glenmark, and Purdue. The result of these efforts has been the identification of many novel and potent TRPV1 antagonists. A select group of these compounds is shown in TABLE 3a,b.

In general, significant *in vitro* and/or *in vivo* data exist for these examples in peer-reviewed publications such that a picture of TRPV1-antagonist effects is beginning to emerge. These compounds and their analogues also provide the basis for a developing structure–activity relationship (SAR) for small-molecule TRPV1 antagonists. For entries 1–9 in TABLE 3a,b, this can be generalized as a central hydrogen-bond acceptor/donor motif flanked by a lipophilic side chain on one side and an aromatic group that incorporates a hydrogen-bond acceptor on the other side (FIG. 4). In TABLE 3a,b, entries 2–7 incorporate structural features that can be traced back to the prototypical TRPV1 antagonist capsazepine (entry 1 in TABLE 3a). Further refinements of the urea/amide motif, such as restricting the number of accessible conformations, have provided examples such as the quinazoline and benzimidazole analogues shown in TABLE 3b (entries 8 and 9, respectively), which are further removed structurally from capsazepine but still retain the key binding elements outlined in FIG. 4. Other TRPV1 antagonists have emerged that do not fit as readily into this model (for example, entry 10 in TABLE 3b). Selectivity data is available for some of the compounds in TABLE 3a,b; in specific cases, broad receptor screening by radioligand binding has been reported (see TABLE 3a,b for references). It should be noted, however, that there is little data available on selectivity as assessed in functional assays. So, for example, although we know that BCTC (*N*-(4-tertiarybutylphenyl)-4-(3-chloropyridin-2-yl)tetrahydropyrazine-1(2H)-carboxamide) is a functional inhibitor of TRPM8 (TABLE 3a) its effects on other ion channels are unclear[84].

The progress of small-molecule drug discovery is also evident in the initiation of clinical trials of TRPV1 antagonists. Amgen, GlaxoSmithKline and Neurogen/Merck have advanced TRPV1 antagonists into clinical testing (TABLE 1). Amgen reported initiation of Phase I trials of AMG517 in September 2004. GlaxoSmithKline's SB-705498 (TABLE 3b) is in Phase I trials for dental pain (SB-705498 Dental Pain Study) and reached Phase II for acute migraine headache (Use Of SB-705498 In The Acute Treatment Of Migraine). In May 2006, GlaxoSmithKline presented Phase I clinical data of SB-705498 in healthy volunteers[85]. SB-705498 significantly reduced capsaicin-evoked flare and acute heat-evoked pain on non-sensitized skin. Furthermore,

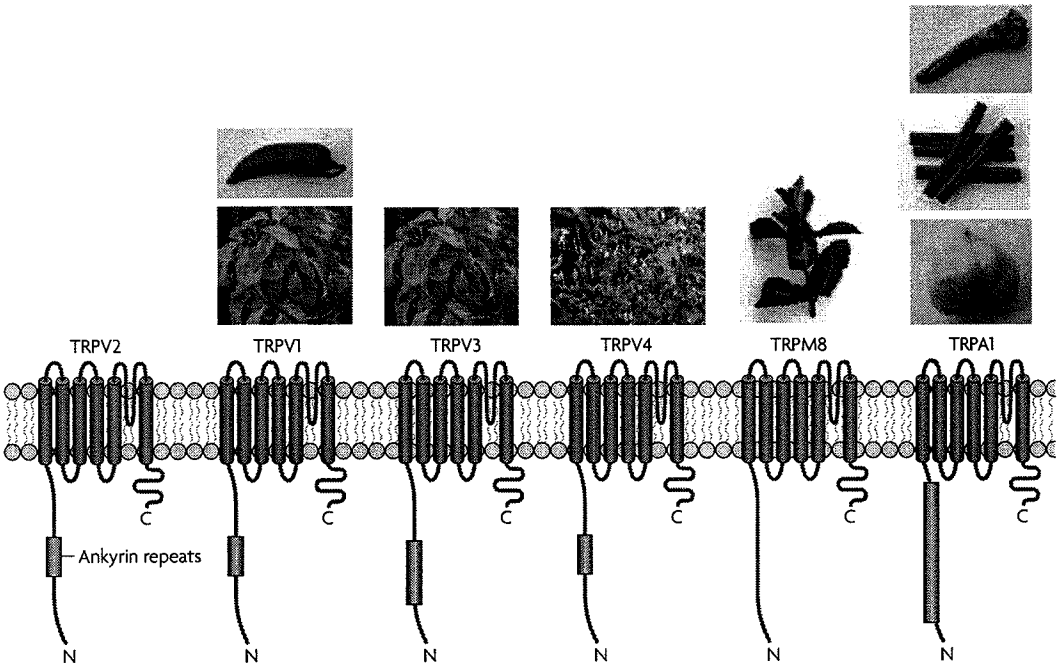


Figure 3 | **Activation of thermoTRPs by naturally occurring compounds.** Schematic depiction of the predicted membrane topology of the thermoTRPs and their activation by natural ligands. These channels are thought to have six transmembrane domains with a proposed pore region between segment 5 and 6. The amino and carboxy termini are cytoplasmic. Channels with ankyrin repeats in their amino termini are indicated. In addition to their thermal sensitivity, thermo transient-receptor-potential (thermoTRP) channels are activated by natural compounds. TRP vanilloid subfamily, member 1 (TRPV1) is activated by capsaicin, which is responsible for the piquancy of hot-chili peppers; TRP melastatin subfamily, member 8 (TRPM8) by menthol, the active ingredient in green mint; TRP ankyrin subfamily, member 1 (TRPA1) by pungent compounds such as cinnamaldehyde, isothiocyanates and allicin, active ingredients in cinnamon, horseradish and garlic, accordingly; TRPV1 and TRPV3 by camphor, isolated from the wood of the camphor laurel tree (*Cinnamomum camphora*); and TRPV4 by bisandrographolide, present in the Chinese herbal plant *Andrographis paniculata*. Photograph of *Adrographis paniculta* © Kazuo Yamasaki, Teikyo Heisei University, Japan.

SB-705498 reduced heat-evoked pain after ultraviolet B (UVB)-evoked inflammation. In November 2006, Neurogen/Merck announced the initiation of Phase II trials with NGD-8243/MK-2295 in acute pain.

The advance of compounds into Phase II clinical trials suggests that TRPV1 antagonists exhibit a benign profile in laboratory animals that are used for preclinical toxicology studies. This speculation is consistent with the unremarkable phenotype of *TRPV1*$^{-/-}$ mice under 'normal' physiological conditions[11,12] — although they do exhibit increased urine voiding or 'spotting' behaviour[86]. There is evidence for an endogenous TRPV1 'tone' in the regulation of core body temperature[87]. Acute systemic capsaicin administration results in a rapid drop in body temperature[88], whereas acute systemic TRPV1 antagonist treatment manifests in a significant increase in body temperature[87]. However, no difference in circadian body temperature fluctuation is observed in *TRPV1*$^{-/-}$ mice versus wild-type mice[89], so the effect of chronic treatment with TRPV1 antagonists in this regard is unclear. Nonetheless, *TRPV1*$^{-/-}$ mice show an attenuated fever in response to lipopolysaccharide (LPS)[89].

One might speculate that TRPV1 antagonists will affect certain physiological processes because of the distribution of TRPV1-containing sensory nerve fibres in a multitude of tissues, the expression of TRPV1 in non-sensory neuron cells and the regulation of TRPV1 expression by ongoing pathophysiology. The potential impact of TRPV1 antagonists in disease states, including pain, is considered below.

Effects of TRPV1 antagonists on the skin and musculoskeletal systems. In the skin, the neurogenic inflammatory response includes vasodilation and edema (flare response), which are mediated by CGRP and SP, respectively[7]. However, in the knee joint of the mouse, capsaicin evokes an unexpected vasoconstrictor effect, the mechanism of which is unclear[90]. Consistent with the hypothesis that TRPV1 activation is a crucial event in C-fibre nociceptor activation under inflammatory conditions, *TRPV1*$^{-/-}$ do not exhibit thermal hypersensitivity in response to an acute hind-paw injection of pro-inflammatory agents, such as CFA or carrageenan[11,12]. These observations have been recapitulated in rats and guinea pigs, which were administered with the TRPV1 antagonists, capsazepine, BCTC, A-425619, AMG9810, compound 46ad and compound 26 (entries 9 and 10 in TABLE 3b). In a CFA model of experimental arthritis, joint swelling is attenuated, but not absent, in *TRPV1*$^{-/-}$ mice, which indicates that mechanisms other than TRPV1 are

Table 2 | **Function of thermoTRP channels and their relevance to pain**

Channel	Thermal threshold	Function and phenotype	Gene-targeted deletion	References
TRPV1	≥43°C	Involved in noxious heat detection and mediates thermal hyperalgesia under inflammatory conditions	Two independent knockout studies	1, 11, 12
TRPA1	≤17°C	Involved in noxious cold detection, mechanical and mustard oil- and bradykinin-induced hyperalgesia	Two independent knockout studies	63, 172, 177
TRPV2	≥53°C	Responds to noxious heat in heterologous systems; upregulated during inflammation	Not reported so far	58, 152
TRPV3	≥33°C	Involved in warm and noxious heat detection	One study	59, 154, 157
TRPV4	≥25°C	Involved in warm temperature sensation; controversial reports about its involvement in mediating noxious heat pain and thermal hyperalgesia	Two independent knockout studies	60, 158–164
TRPM8	≤23°C	Role in the detection of innocuous and noxious cold sensations remains to be determined	Not reported so far	61, 62

TRPA1, transient receptor potential subfamily ankyrin, member 1; TRPM8, TRP subfamily melastatin, member 8; TRPV1,2,3,4, TRP subfamily vanilloid, member 1,2,3 or 4.

also involved in the development of joint inflammation[91]. Importantly, histology showed no major differences between TRPV1 wild-type and knockout mice, which suggests that TRPV1 is not involved in the development of joint abnormalities[91]. In a mouse model of cancer pain, systemic administration of the TRPV1 antagonist JNJ-17203212 (TABLE 3a) results in efficacy according to several behavioural measures of pain. *TRPV1*$^{-/-}$ mice show a similar extent of efficacy as *TRPV1*$^{+/+}$ controls that were treated with the antagonist[92].

Interestingly, *TRPV1*$^{-/-}$ mice develop mechanical hypersensitivity to the same extent as wild-type mice[11,12]. By contrast, TRPV1-antagonist treatment alters mechanical hypersensitivity induced by inflammation or nerve injury in rats[91,93–96]. This incongruous result might be explained by 'off-target' activities of the agents used in these studies. In fact, capsazepine inhibits voltage calcium channels[97] and nicotinic acetylcholine receptors[98], and BCTC inhibits TRPM8 activity with high affinity (with an IC_{50} of 143 nM)[84]. But poor selectivity seems an unlikely explanation given the structural diversity of the reported antagonists. For instance, stretch sensitivity in colonic afferents is reduced in *TRPV1*$^{-/-}$ mice, which provides experimental support for the involvement of TRPV1 in aspects of mechanical-force-sensitive signalling[99]. Moreover, TRPV1 sensitization might underlie an increase in generator potential in nociceptors, therefore driving an increased spontaneous action-potential firing in these cells post-injury[15]. If so, traditional preclinical pain models, which rely on evoked withdrawal thresholds, might underestimate the true analgesic potential of TRPV1 antagonists. Indeed, A-425619 gives statistically significant reversal of the hind-limb weight-bearing differential that is induced by the unilateral injection of sodium monoiodoacetate into the knee joint of rats[94]. Furthermore, JNJ-17203212 was judged efficacious in mouse cancer pain on the basis of, in part, improvements in spontaneous flinching and guarding of the affected hind-limb[94]. These data suggest that constant spontaneous pain, sometimes referred to as burning pain, might be positively affected by TRPV1 antagonist administration.

Effect of TRPV1 antagonists on the GI system. TRPV1-immunoreactive fibres constitute over half of the sensory afferents that project into the viscera (reviewed in REF. 80). Most of the TRPV1-containing innervation of the GI system is of spinal origin. Elevated TRPV1 immunoreactivity has been observed in colonic sensory nerve fibres in patients with IBD[71], in rectal sensory nerve fibres in patients with rectal hypersensitivity and faecal urgency[72], and in oesophageal mucosa sensory fibres in patients with gastroesophageal reflux disease[100]. Therefore, a clear hypothesis for the use of TRPV1 antagonists in treating the pain that results from GI disease exists and, as reviewed elsewhere[80], numerous preclinical studies have explored the role of TRPV1 in the gut. Although it is unclear whether TRPV1 antagonists administered therapeutically can reverse ileitis-induced pain based on preclinical models, systemic capsazepine decreases nociceptor signalling as assessed by c-fos staining in an L-arginine-induced pancreatitis model[101], and decreases markers of inflammation in multiple GI-inflammation models including caerulein-induced pancreatitis in rats[102]. Capsazepine also decreases physiological responses to colorectal or jejunal distension in mice[103], an effect comparable to observations in *TRPV1*$^{-/-}$ mice[99]. A limitation in our understanding of TRPV1 antagonist utility in treating disorders of the GI system is that capsazepine is the only agent studied in these preclinical models so far. However, the extent of TRPV1 expression in sensory neurons and its potential to contribute to spontaneous firing strongly suggest that TRPV1 antagonists will be useful agents to treat GI-disease pain.

Effect of TRPV1 antagonists on the urinary system. The few published reports that describe the effects of TRPV1 antagonists in preclinical models of bladder hyperreflexia give tantalizing pieces of data that suggest a TRPV1-antagonist use in bladder hyperreflexia states. As reviewed recently, instillation of capsaicin or RTX results in decreased micturition frequency presumably because of a desensitization of the sensory neuron fibres that innervate the urinary bladder[104]. Indeed, TRPV1

Box 1 | **Warm and hot-temperature-activated thermoTRPs**

TRPV2. Transient receptor potential vanilloid subfamily, member 2 (TRPV2) responds to noxious heat at temperatures that exceed 53°C (REF. 58) and is upregulated under inflammatory conditions[153]. So far, no null-mutant mice of TRPV2 have been reported and its therapeutic potential or role in noxious stimuli detection remains to be evaluated.

TRPV3. TRPV3 is expressed in dorsal root ganglia and keratinocytes where it may act as a heat sensor and responds to warm temperatures (33–39 °C)[59,154,155]. Interestingly, TRPV3 strongly sensitizes to multiple applications of heat and/or sensitizers such as camphor, 2-APB (2-aminoethyl diphenylborate), carvacrol, thymol and eugenol, indicating a potential role in nociception[156–158]. Indeed, *TRPV3*-mutant mice demonstrate a deficit to noxious acute thermal stimulation at temperatures ≥ 50°C (REF. 158). In contrast to TRPV1 (REFS 11,12), *TRPV3*-mutant mice showed normal behaviour in models of inflammatory pain suggesting that TRPV3 may not be of therapeutic value for such indications[158].

TRPV4. TRPV4 is expressed in the skin and dorsal root ganglion neurons and responds to warm temperatures (25–34°C)[60,159]. Temperature and other TRPV4 modulators, such as anandamide, arachidonic acid, epoxyeicosatienoic acid and α-phorbol didecanoate (PDD) work together to activate the channel[160]. Recently, it was shown that bisandrographolide A from the Chinese herbal plant *Andrographis paniculata* activates TRPV4 (REF. 161). In addition to a deficit in osmoregulation, *TRPV4*-mutant mice exhibited a higher threshold to intense mechanical stimulation[162,163]. Surprisingly, *TRPV4*-mutant and wild-type mice behaved normally in the hot-plate assay (up to 50°C) or when exposed to radiant heat, suggesting no function in acute thermal sensation[164]. By contrast, *TRPV4*-mutant mice exhibit higher withdrawal latency in response to heat applied to their tail (45–46°C)[165]. *TRPV4*-mutant mice behaved normally in temperature gradient assays after intraplantar complete Freund's adjuvant injection; other studies, however, suggested that TRPV4 has an essential role in models of carrageenan-induced thermal and inflammatory mediator-induced mechanical hyperalgesia[164,166]. Furthermore, spinal administration of antisense oligodeoxynucleotides to TRPV4 abolished mechanical hyperalgesia in a model of taxol-induced neuropathic pain[167]. Given the inconsistencies in these studies, the role of TRPV4 in inflammatory pain remains unclear.

TRPM2, TRPM4 and TRPM5. Transient receptor potential melastatin subfamily, member 2 (TRPM2) was recently shown to be activated by warm temperatures (≥ 35 °C) apparently by direct gating of the channel[168]. Although β-NAD^+ or ADP-ribose are known agonists of TRPM2, the activity of the channel is enhanced by co-application of heat[168]. TRPM4 (REF. 66) and TRPM5 (REF. 67) are non-selective cation channels that are impermeable to calcium and are activated by elevated levels of intracellular calcium. Both channels can be activated by bringing the temperature to between 15 and 35°C. TRPM5 is indirectly involved in the transduction of taste in which it is thought to be activated downstream of taste receptor type 1, member 1 (T1R), T2R and phospholipase C-β2 (PLCβ2) by elevated calcium in the cytosol[169]. Although TRPM2, TRPM4 and TRPM5 are sensitive to heat, no expression in sensory neurons was reported for these channels. Therefore, their function in thermosensation or pain remains unknown.

immunoreactivity has been detected in afferents in human bladder biopsies and increased immunoreactivity has been correlated with neurogenic detrusor overactivity[105]. Intriguingly, instillation of RTX or botulinum neurotoxin type A, both of which are effective treatments for detrusor overactivity, decrease the extent of TRPV1 immunoreactivity in the bladder[105,106]. Direct application of capsazepine to the bladder decreases micturition frequency in cyclophosphamide-treated, but not control, rats under anesthaesia[107]. By contrast, $TRPV1^{-/-}$ mice demonstrated reduced voiding reflex in response to controlled filling under anesthaesia, but also demonstrate increased spotting or voiding frequency while conscious, relative to wild-type mice[86]. The apparent differences between the capsazepine and knockout studies might be related to species differences in bladder physiology. Nonetheless, these studies raise the possibility that TRPV1 antagonists can effect an alteration in voiding frequency.

Effect of TRPV1 antagonists on the respiratory system. In human airways, TRPV1 is believed to represent an important target for toxicants[108]. As reviewed elsewhere[109], SP released from capsaicin-sensitive neurons mediates robust bronchoconstriction through neurokinin 2 (NK2; also known as TACR2) receptors and stimulates seromucous secretion from bronchial glands by interacting with NK1 (also known as TACR1) receptors. These changes attract inflammatory cells that, in turn, release proteases such as trypsin and tryptase. These enzymes activate protease-activated receptor 2 (PAR2)[110], which further stimulates TRPV1 through a PKCε-dependent mechanism[111], causing a positive-feedback loop that results in increased vanilloid and citric acid-induced cough in guinea pigs[112].

Collier and Fuller first described the cough-inducing effects of capsaicin in a clinical setting[113]. This work clearly shows that the activation of sensory fibres is necessary for the cough response. Capsaicin also reduces airway conductance in humans, and the dose of capsaicin required to cause a cough is reduced in patients with asthma and chronic obstructive pulmonary disease (reviewed in REF. 109). Similarly, patients with a chronic cough show increased tussive sensitivity to capsaicin and have higher levels of TRPV1 immunoreactivity in airway sensory fibres[114]. As elevated bradykinin levels are mechanistically involved in angiotensin-converting enzyme (ACE)-inhibitor-induced chronic cough, further mechanistic support for the role of TRPV1 in coughing comes from the observation that bradykinin stimulates airway afferents in a TRPV1-dependent manner[115]. Not surprisingly, capsazepine and I-RTX have been shown to block capsaicin and citric acid-induced cough responses in guinea pigs[116,117], and BCTC attenuates cough responses to ovalbumin antigen in ovalbumin-sensitized guinea pigs[118]. As capsaicin and citric acid are widely used for clinical experimental medicine purposes[119], and as the guinea pig is a well-established preclinical species for the study of coughing[120], these results demonstrate the potential for TRPV1 antagonists to be clinical anti-tussives[121].

Effects of TRPV1 antagonists on the vascular system. BIBN-4096BS, also known as olcegepant, is a potent CGRP-receptor antagonist that blocks the vasodilator effects of CGRP and exhibits efficacy in patients with migraine and cluster headache[122]. As CGRP is strongly co-expressed in many TRPV1-expressing nerve fibres, including sensory fibres that innervate the dural vasculature[123], it is plausible to consider the possibility that activation of TRPV1 could partially underlie a neurogenic-mediated component of headache. Indeed, TRPV1 might be activated by mediators that are released from the vasculature during a headache[124]. For example, anandamide that is released from the endothelium can activate TRPV1 that is expressed on trigeminal afferents in the cerebrovasculature[125]. Unfortunately, little information is available on the efficacy of TRPV1 antagonists in preclinical models of headache.

The co-expression of CGRP and TRPV1 also implies other vascular effects of TRPV1 modulation. CGRP that is administered intravenously has a hypotensive

Box 2 | **Cool and noxious cold-temperature-activated thermoTRPs**

TRPM8. Transient receptor potential melastatin subfamily, member 8 (TRPM8) responds to cool temperatures with an activation threshold of 23–27°C (REFS 62,63). Interestingly, compared with TRPV1 (TRP vanilloid subfamily, member 1), TRPM8 shows the opposite mechanisms of activation. For instance, TRPV1 is sensitized by heat, acidification and protein kinase C (PKC), whereas TRPM8 exhibits a decrease in its activity in response to these agents. TRPM8 is believed to function as an innocuous cool receptor. In agreement with this, TRPM8 expression is restricted to a subset of dorsal root ganglia and trigeminal neurons that do not express known markers of nociception[62,63]. Although TRPM8 and TRPV1 clearly define distinct subpopulations of sensory neurons *in vivo*[170], there is an overlap in their functional expression in cultured and acutely dissociated dorsal root ganglion neurons using capsaicin and menthol as agonists[171]. Mutant mice lacking in TRPM8 have yet to be reported. The generation of such mice should ultimately define the role of TRPM8 in the detection of innocuous and noxious cold sensations.

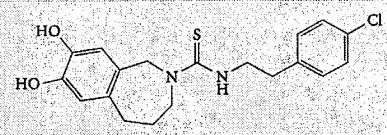
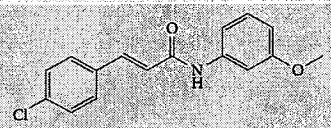
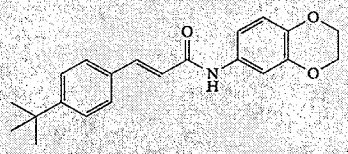
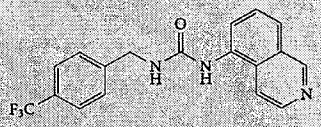
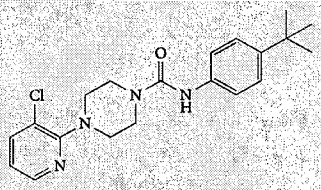
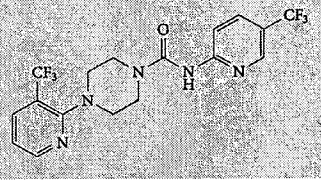
TRPA1. Transient receptor potential ankyrin subfamily, member 1 (TRPA1) is activated by noxious cold temperatures (≤ 17 °C), and is co-expressed with TRPV1 on sensory neurons[64]. TRPA1 is also activated by pungent compounds and irritants such as cinnamaldehyde, isothiocyanate, allicin and acrolein (a metabolized byproduct of chemotherapeutic agents that is also present in tear gas and vehicle exhaust)[172–176]. Recent studies have suggested that many of the structurally diverse TRPA1 agonists (most of which are electrophilic in nature) activate the channel through covalent modification of reactive cysteine residues within the receptor[177]. Two independent knockout studies showed that *TRPA1*-mutant mice did not develop thermal and mechanical hyperalgesia after intraplantar injection of bradykinin and mustard oil[172,178]. *TRPA1*-mutant mice also showed reduced sensitivity to intense cold stimulation and a higher threshold of activation in response to painful punctuate mechanical stimulation[178]. In a spinal nerve-ligation model (SNL), TRPA1 mRNA expression was increased in the nearby uninjured L4 dorsal root ganglia. Furthermore, antisense knockdown of TRPA1 suppressed cold hyperalgesia in SNL rats[179]. TRPA1 antagonism might prevent acrolein side effects resulting from cyclophosphamide- or ifosfamide-based chemotherapy. Finally, TRPA1 may also serve as a pharmacological target for pulmonary oedema and respiratory irritation caused by environmental irritants related to the acrolein class[172].

effect[126], and has been suggested to confer a beneficial counterbalance to the development of hypertension[127]. Intravenous injection of capsazepine increased systolic blood pressure in Dahl salt-resistent rats that were maintained on a high-salt diet for 3 weeks[128]. By contrast, Dahl salt-sensitive rats on a high-salt diet, which exhibit significantly increased systolic blood pressure, are unaffected by capsazepine treatment[128]. Intriguingly, cations can sensitize TRPV1 currents and activate nociceptive signalling by elevated ionic strength, which suggests a role for TRPV1 in mediating physiological responses to high salt. Moreover, *TRPV1*$^{-/-}$ mice exhibited a decreased serum arginine-vasopressin response to increased serum osmolarity, which was induced by high-salt intake. It should be noted, however, that no alteration in baseline cardiovascular parameters is observed in *TRPV1*$^{-/-}$ mice, although the Bezold–Jarisch reflex (increased vagal, parasympathetic, efferent discharge to the heart elicited by stimulation of chemoreceptors), which is initiated by high plasma concentrations of anandamide (20 mg per kg, intravenously) is decreased in *TRPV1*$^{-/-}$ mice versus *TRPV1*$^{+/+}$ controls[129]. Taken together, these results suggest that TRPV1 might be involved in the pressor response to high serum-sodium concentrations, although the related channel TRPV4, which is known to be sensitive to osmolarity changes and is expressed in the hypothalamus and kidney, has also been implicated in high salt-induced responses[130].

TRPV1 might be involved in mediating a sensory nerve fibre response to ischaemia, which could include neurogenic release of CGRP. However, the literature that describes the physiological effect of CGRP and TRPV1 on the cardiovascular system response to ischaemia is filled with contradictions. Several studies demonstrate no effect of the CGRP antagonist CGRP(8–37) on acute myocardial ischaemia-induced changes in coronary arteriole microvessel diameter in dogs or on myocardial ischaemia-induced infarct size in pigs[131,132]. These studies observed decreased mean aortic and arterial pressure, respectively, in animals that were administered with high doses of exogenous CGRP. The studies also observed CGRP(8–37)-mediated inhibition of the exogenous CGRP effect, which suggests that endogenous CGRP has little effect on coronary vascular tone during ischaemia. Moreover, studies in rats with the CGRP antagonist BIBN-4096BS demonstrate that blockade of CGRP has no detrimental effect after a 60 min myocardial ischaemia-induced infarct size in rats, even though plasma concentrations of CGRP were increased by 50% during ischaemia[133]. However, one study indicates that ischaemic preconditioning — a brief coronary artery occlusion, which has been shown to provide some protection against damage caused by a subsequent prolonged occlusion — is mediated by endogenous CGRP[134]. This is based on the observation that pretreatment of rats with BIBN-4096BS before ischaemic preconditioning (15 min coronary artery occlusion) results in greater myocardial-infarct size and creatine-kinase release than controls in response to prolonged ischaemia (45 min coronary artery occlusion)[134]. A study using the Langendorff apparatus reported that hearts from C57BL/6J mice that were treated with capsazepine before 40 min of no-flow global ischaemia exhibited decreased cardiovascular performance after reperfusion (increased left-ventricular developed pressure and coronary flow) in comparison with hearts from control mice[135]. Identical experiments in *TRPV1*$^{-/-}$ mice generated results similar to those of capsazepine-treated *TRPV1*$^{+/+}$ mice[135]. However, these investigators observed no effect of CGRP(8–37) on post-ischaemic recovery in similar experiments. Because the preclinical data set contains contradictions on many levels (for example, the lack of significant effects of CGRP(8–37) in pigs, dogs and mice versus the effects of capsazepine and *TRPV1*$^{-/-}$ genotype in mice versus the contradictory findings with BIBN-4096BS), an understanding of TRPV1 antagonist effect in ischaemia is challenging.

Another role for TRPV1 in the sensory nerve fibre response to ischaemia might be the transduction of pain signals that originate from mediators that are released by damaged tissue. In one study, all cardiac afferents in ferrets, which exhibited increased spontaneous firing activity in response to 5 min myocardial ischaemia (coronary artery occlusion), were all judged to be C-fibres[136]. Following a 15–20 min recovery period, the receptive fields of these fibres on the epicardial surface were treated with I-RTX. A second 5 min myocardial ischaemia challenge revealed a significant decrease in spontaneous firing[137]. This observation suggests an

Table 3a | **Summary of important TRPV1 antagonists**

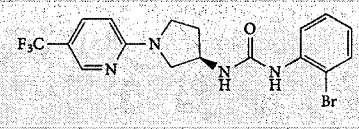
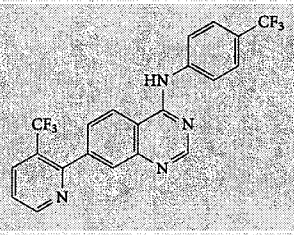
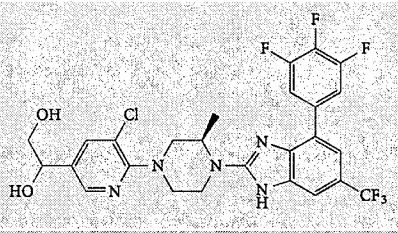
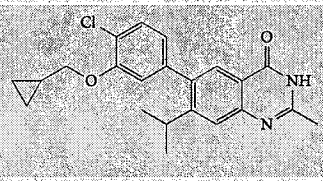
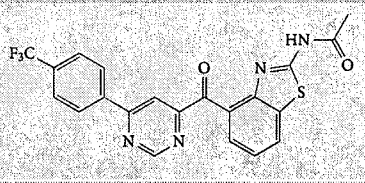
Name	Structure	Comments	References
Capsazepine (thiourea)		• rIC_{50} = 420 nM ($^{45}Ca^{2+}$ uptake) • Inhibits voltage-activated calcium channels and nicotinic acetylcholine receptors • Significantly reversed CFA-induced mechanical hyperalgesia in guinea pigs	180 97, 98 96
SB-366791 (cinnamide analogue)		• hK_i = 18 nM (FLIPR) • Selective versus TRPV4 and other TRP channels • Inhibits capsaicin and heat-mediated activation of TRPV1	182
AMG-9810 (cinnamide analogue)		• hIC_{50} = 25 nM ($^{45}Ca^{2+}$ uptake) • hIC_{50} of > 4 μM at TRPV3, TRPV4, TRPA1 and TRPM8 • Inhibits CFA-induced thermal (30 mg per kg) and mechanical hyperalgesia (100 mg per kg)	183
A-425619 (urea analogue)		• hIC_{50} = 5 nM (FLIPR) • TRPM8 IC_{50} = 8 μM; TRPA1 IC_{50} > 10 μM • Inhibits CFA-induced thermal hyperalgesia (ED_{50} = 10 mg per kg)	183 94
BCTC (urea analogue)		• hIC_{50} = 35 nM (FLIPR) • TRPM8 IC_{50} = 143 nM • Inhibits CFA-induced thermal and mechanical hyperalgesia (3–30 mg per kg, orally) • Reduces tactile allodynia and thermal hyperalgesia in a partial nerve-ligation model	184 84 95
JNJ-17203212 (urea analogue)		• hIC_{50} = 65 nM (FLIPR) • Elicits ~1°C increase in core body temperature in rats (30 mg per kg, orally) • Attenuates nocifensive behaviours in an *in vivo* model of bone-cancer pain	87 92

BCTC, *N*-(4-tertiarybutylphenyl)-4-(3-chloropyridin-2-yl)tetrahydropyrazine-1(2H)-carboxamide; CFA, complete Freund's adjuvant; ED_{50}, half-maximal effective dose; FLIPR, fluorescence imaging plate reader; hIC_{50}, half maximal inhibitory concentration in humans; hK_i, inhibiton constant in humans; rIC_{50}, half maximal inhibitory concentration in rats; TRP, transient receptor potential receptor; TRPA1, TRP subfamily ankyrin, member 1; TRPM8, TRP receptor subfamily melastatin, member 8; TRPV1,3,4, TRP receptor subfamily vanilloid, member 1,3 or 4.

important role for TRPV1 in sensory afferent response to transient ischaemia. However, earlier work in rats indicated that the bradykinin-induced cardiac-sympathetic reflex (increased renal-sinusoidal nerve activity and increased mean arterial pressure), although mediated in part by TRPV1-expressing sensory fibres, was not dependent on TRPV1 (REF. 137). So, although cardiac sensory fibres have a role in mediating chest pain and associated autonomic reflexes, TRPV1 is not the only channel or receptor suggested to have a role in mediating sensory neuron responses to ischaemia; these channel and receptors include acid-sensing ion channel 3 (ASIC3; also known as ACCN3), TREK1 (also known as KCNK2) and bradykinin receptors.

Effects of TRPV1 antagonists on the CNS. Histochemical studies have confirmed the expression of TRPV1 in the CNS, including the hypothalamus and substantia nigra[81]. As mentioned previously, the hypothalamic supraoptic nucleus expresses an N-terminal variant of TRPV1, which is important in the arginine-vasopressin responses to high serum osmolarity[58]. Hypothalamic expression also helps to explain the hypothermia that is induced by injection of capsaicin into the hypothalamus[138]. Conversely, systemic administration of a TRPV1 antagonist (JNJ-17203212) to rats results in transient hyperthermia of about 1°C (REF. 87). This unanticipated result suggests that a TRPV1 tone exists, possibly in the anterior hypothalamic area, which is involved in the setting and sensing of core body temperature. *TRPV1*$^{-/-}$ mice exhibit core body temperatures that are identical to the wild-type mice[89], observations that are corroborated by experiments in which rats have been given a TRPV1 antagonist chronically (R. K. Conley, S. Boyce and D. N. Cortright, unpublished observations). Intriguingly, *TRPV1*$^{-/-}$ mice demonstrate a significantly smaller

Table 3b | **Summary of important TRPV1 antagonists**

Name	Structure	Comments	References
SB-705498 (urea analogue)		• rIC_{50} = 32 nM (FLIPR) • Phase I: reduced capsaicin-evoked flare and acute heat-evoked pain on non-sensitized skin	185 85
Quinazoline analogue		• hIC_{50} = 1 nM (FLIPR) • Achieved 80% block of carrageenan-induced thermal hyperalgesia at 3 mg per kg (MED 0.1 mg per kg)	186
Compound 46ad (benzimidazol analogue)		• hIC_{50} = 1 nM ($^{45}Ca^{2+}$ uptake) • Achieved significant reversal of CFA-induced thermal hyperalgesia (30 mg per kg, orally)	187
Compound 26 (quinazolinon analogue)		• hIC_{50} = 50 nM (low pH activation) • Achieved 60% reversal of CFA-induced mechanical hyperalgesia (30 mg per kg, orally) • Achieved 57% reversal of mechanical hyperalgesia in a partial nerve-ligation model	93
AMG 517		• Initiation of Phase I clinical trials reported in September 2004 • hIC_{50} = 0.9 nM ($^{45}Ca^{2+}$ uptake) • Achieved ~40% block of CFA-induced thermal hyperalgesia at 10 mg per kg (MED 1 mg per kg)	M. Norman, personal communication
NGD 8243	Undisclosed	• Initiation of Phase II trials announced in November 2006	Neurogen, press release

CFA, complete Freund's adjuvant; FLIPR, fluorescence imaging plate reader; hIC_{50}, half maximal inhibitory concentration in humans; hK_i, inhibiton constant in humans; MED, minimum effective dose; rIC_{50}, half maximal inhibitory concentration in rats.

febrile response to injection of bacterial LPS than wild-type mice[88], although LPS-induced fever in rats is not affected by capsazepine[139].

The observation that I-RTX significantly reduces the frequency of spontaneous excitatory post-synaptic currents in substantia nigra pars compacta dopaminergic neurons *in vitro* provides additional evidence for an endogenous TRPV1 tone in the CNS[140]. Additionally, TRPV1 activation of dopaminergic mesencephalic neurons *in vitro* (by high concentrations of capsaicin or anandamide) and *in vivo* (by intranigral injection of capsaicin or anandamide) results in cell death[141]. These data might imply a neuroprotective role for TRPV1 antagonists on these neurons although this result has not yet been corroborated. The functional effect of TRPV1 on thermoregulation and, possibly, on dopaminergic neurons, supports the hypothesis that TRPV1 is functionally expressed in the brain[9,142], but additional experiments are necessary to understand the full effect of TRPV1 antagonists on CNS processes.

An emerging role for TRPV1 in glucose regulation

Several lines of evidence imply a role for TRPV1 in the regulation of plasma glucose levels. A dense meshwork of sensory nerve fibres is found in the pancreas[143], and TRPV1 is also expressed on pancreatic islet cells where it is thought to have a role in insulin release[144]. Interestingly, insulin sensitizes TRPV1 on sensory nerve endings[145], thereby creating a local feedback interaction between islet cells and primary sensory neurons that innervate the Langerhans islets. The afferent arm of this feedback loop is composed of neuropeptides, in particular CGRP, that were shown to reduce insulin release from β cells[146,147]. SP that is released from TRPV1-expressing nerves promotes neurogenic inflammation in the pancreas[102].

It is well documented that ablation of TRPV1-positive neurons by agonist (capsaicin or RTX) administration in rat neonates improves glucose tolerance in those made diabetic by streptozotocin treatment[148]. This observation is not unexpected as neonatal capsaicin

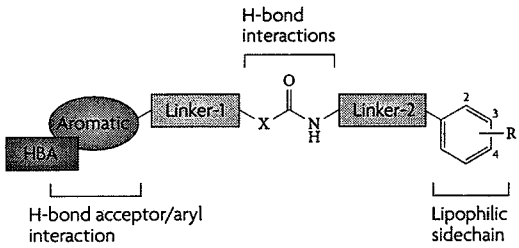


Figure 4 | **Key binding interactions of TRPV1 antagonists.** These are based on entries 1–9 in TABLE 3a,b and related structures. The indicated hydrogen-bonding motif is present in most known transient receptor potential vanilloid subfamily, member 1 (TRPV1) antagonist structures. Both H-bond donor and acceptor tend to be important for optimal potency. These are readily provided by urea (X=N), thiourea, amide (X=C) or reverse-amide functionalities, among others. Mono- or bicyclic-aryl and heteroaryl rings with a properly positioned hydrogen-bond acceptor (HBA) in this part of the molecule improve both potency and drug-like properties. Interactions (π–π) between this aryl ring and another on TRPV1 are possible, as has been suggested for the agonist capsaicin. The lipophilic side-chain interacts with a hypothetical hydrophobic binding site on TRPV1. Proper placement of lipophilic substituents (often 4-CF_3 or 4-t-Bu) is crucial for optimal TRPV1 potency. The linkers serve as scaffolding for the proper positioning/spacing of the three interactions above and, therefore, can take many forms, such as direct bonds, single-atom or double-atom spacers or ring systems.

treatment depletes CGRP (reviewed in REFS 2,4), a neuropeptide that would antagonize insulin release[147].

Treatment of obese Zucker rats, a genetic model of type 2 diabetes, with desensitizing doses of capsaicin or RTX was shown to result in significantly decreased fasting-plasma insulin levels, improved glucose tolerance through enhancement of insulin secretion and increased glucose infusion rate during euglycemic hyperinsulinaemic clamp versus vehicle controls[149]. That is, RTX treatment results both in increased insulin secretion and sensitivity, which suggests that TRPV1-expressing cells might be involved in glucose regulation. Whether the effects of desensitizing vanilloid agonist treatment can be recapitulated with TRPV1 antagonist treatment has yet to be demonstrated.

New findings obtained in non-obese diabetic (NOD) mice, which are genetically prone to develop type 1 (insulin-dependent) diabetes, imply a more important role for TRPV1 in the development of diabetes than previously thought[150]. In these animals, ablation by neonatal capsaicin treatment of TRPV1-positive neurons that innervate the pancreas (NOD^{CAPS} mice) prevents insulitis and resulting β-cell destruction that would ultimately result in type 1 diabetes, despite the systemic persistence of pathogenic T lymphocytes. Apparently, these mice carry a hypofunctional TRPV1 mutant ($TRPV1^{NOD}$) that is localized to the *Idd4.1* diabetes-risk locus. As expected, nociceptive behaviour in response to capsaicin is markedly depressed in NOD mice compared with non-obese diabetic-resistant (NOR) mice with wild-type TRPV1. Capsaicin treatment causes a dramatic reduction in insulitis without having a noticeable effect on autoimmune infiltrations elsewhere in the NOD mice. It is puzzling why the protective capsaicin effects are pancreas-specific in the NOD animals. Another unanswered question is the nature of the substance that mediates the protective action. Intra-arterial injection of SP into the NOD pancreas reverses insulitis and insulin resistance for weeks. This is an unexpected finding as neonatal capsaicin depletes, and not elevates, SP levels (reviewed in REFS 2,4), and endogenous SP that is released from TRPV1-positive fibres promotes neurogenic inflammation[102]. It is also unclear why the congenic NODxB6*Idd4* mice, that have wild-type TRPV1, are diabetes-resistant, similar to the NOD^{CAPS} animals in which TRPV1 has been chemically ablated[150]. One should, however, keep in mind that NOD mice are born with a hypofunctional TRPV1 and we cannot exclude a developmental compensatory mechanism that would create a permissive environment (an abnormal, pro-diabetogenic TRPV1) for insulitis and destruction of the β cells. This might explain why both the ablation by capsaicin of the abnormal TRPV1 neurons and/or the restoration of wild-type TRPV1 in the congenic animals rescues the phenotype[150].

TRPV1-agonist-based therapies

The lack of thermal hyperalgesia in TRPV1-null mice in models of inflammatory pain[11,12] stimulated the biotechnology and pharmaceutical companies to develop small-molecule antagonists that target TRPV1 as potential analgesic drugs. There is no doubt that such antagonists have completely overshadowed TRPV1 agonists. One should, however, keep in mind that although TRPV1 antagonists are just making their debut in the clinic TRPV1-agonist-based therapies have been used for centuries[2,15,151,152]. As already alluded to above, TRPV1 agonists and antagonists are not equivalent therapeutic approaches. Capsaicin-sensitive nerves express a myriad of receptors that are relevant to pain and inflammation of which TRPV1 is just one. TRPV1 agonists silence the whole nerve terminal, whereas antagonists selectively impair TRPV1 (REFS 2,8,15). Those who favour TRPV1 agonists argue that these compounds should be more powerful analgesic drugs than antagonists as they simultaneously block all receptors on capsaicin-sensitive nerves. By contrast, those who favour TRPV1 antagonists emphasize the initial excitation (pain) by agonist application and the potential for irreversible toxicity. In our opinion, TRPV1 agonists and antagonists are not mutually exclusive: quite the contrary, both therapeutic approaches could find their own niche in clinical practice (TABLE 1).

TRPV1 agonists aim to achieve desensitization of the sensory neurons[2,7]. As reviewed elsewhere, capsaicin-based products are associated with an intolerable burning sensation and the need for multiple applications for weeks to mediate their analgesic effect[2]. The ultrapotent capsaicin analogue RTX seems to be devoid of the above mentioned shortcomings of capsaicin, but its clinical use is riddled with problems of its own. Most importantly, RTX is a complex, highly hydrophobic molecule, expensive

to manufacture and difficult to keep in solution[2]. This might explain the strikingly dissimilar clinical outcomes in various studies that use similar patient populations (reviewed in REF. 104). Clearly, in animal experiments RTX is a highly effective and well-tolerated agent to achieve lasting and fully reversible desensitization (defunctionalization) of capsaicin-sensitive neuronal pathways[2].

Reduced-pungency capsaicin analogues and high-concentration injectable or topically applied capsaicin are being actively pursued in the clinic by companies such as NeurogesX, Winston Laboratories and Anesiva (formerly known as Corgentech). In May 2005, NeurogesX initiated Phase III trials for NGX-4010 (Transacin), a clinically administered trans-capsaicin patch to treat post-herpetic neuropathy. Furthermore, NeurogesX announced in February 2006 the positive Phase III trial results in painful HIV-associated sensory neuropathy. Winston Laboratories is developing WL-1001, an intranasal civamide (cis-capsaicin), which is currently in Phase III trials for cluster headache and Phase II for migraine prophylaxis (H. Fezatte and S. Phillips, personal communication). The company initiated Phase III trials for WL-1002, a topical civamide for treatment of osteoarthritis pain. Anesiva is developing compound 4975, a high-concentration injectable formulation of capsaicin, which is in Phase II clinical trials for the treatment of post-surgical, neuropathic and musculoskeletal-pain syndromes.

TRPV1 antagonists: concluding comments

TRPV1, perhaps the most important signal integrator in sensory nociceptors, is well established as an intriguing novel target for the treatment of pain[2,8,15,151,152]. Extensive preclinical profiling of small-molecule inhibitors of TRPV1 provides intriguing evidence that TRPV1 blockade can be a useful therapeutic approach for inflammatory, cancer and possibly neuropathic pain (reviewed in REFS 15,152). Additional preclinical data indicate that TRPV1 antagonists might provide a useful therapeutic option for urinary incontinence, pancreatitis, cough and migraine headache (recently reviewed in REF. 151). Marketed therapies for these disorders and pain have limitations in safety and/or efficacy that drive the need for novel treatment options. For example, NSAIDs (non-steroidal anti-inflammatory drugs), opiates and other analgesics are useful drugs for many patients, but exhibit dose-limiting side effects, inadequate tolerability profiles and diminished efficacy over time. Consequently, pain is often under treated. Although the extensive distribution of TRPV1-expressing sensory fibres creates potential opportunities for TRPV1 antagonists to affect many physiologies, it is in the treatment of pain that these agents may have the most promise. TRPV1 antagonism represents one of several novel mechanistic approaches to pain relief that might qualify as the next-generation analgesic. But although most of the drugs in development target the inflammatory system and the propagation and transmission of signals to the spinal cord, TRPV1 antagonists target the key mediator of nociceptive transduction. Because of TRPV1's integrative signalling properties in response to inflammatory stimuli, TRPV1 antagonists are predicted to inhibit the sensation of ongoing or burning pain (spontaneous pain) that is reported by patients suffering from chronic pain, therefore, offering an unprecedented advantage in selectively inhibiting painful signalling without mechanistic limitations.

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Competing interests statement

The authors declare competing financial interests: see web version for details.

DATABASES

The following terms in this article are linked online to:

Entrez Gene:

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TRPA1 | TRPM2 | | TRPM4 | TRPM5 | TRPM8 | TRPV1 | TRPV2 | TRPV3 | TRPV4 | TRPV5 | TRPV6

FURTHER INFORMATION

SB-705498 Dental Pain Study:

http://www.clinicaltrials.gov/ct/show/NCT00281684

Use Of SB-705498 In The Acute Treatment Of Migraine:

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CORRIGENDUM

The vanilloid receptor TRPV1: 10 years from channel cloning to antagonist proof-of-concept

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In Table 3b on page 367, there is an error in the structure of the compound AMG 517. The correct structure is shown below.